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COMPONENTS OF NITROGENOUS EXCRETA IN THE EARTHWORMS *LUMBRICUS TERRESTRIS*, L. AND *EISENIA FOETIDA* (SAVIGNY)

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This work was undertaken initially in order to obtain normal standards of comparison for the pattern of nitrogen excretion during wound healing and regeneration in earthworms. The main interest in the results, for the present purpose, lies in the contrast between the patterns of nitrogen output in the two species. The pattern of excretion in *Lumbricus* is quite well known, from the studies of Lesser (1908), Delaunay (1934) and his colleagues, and Cohen & Lewis (1949*a*, *b*, 1950); there is also some information about *Pheretima* (Bahl, 1945, 1947) and about some representatives of other classes of the Annelida (see Prosser *et al.* 1950), but none of these results have anticipated the great contrasts here observed between two rather closely related genera. A certain amount of work has been done also on *Allolobophora caliginosa* (Savigny), but this will not be described in detail.

METHODS

The worms were isolated, individually, into 50 or 100 ml. conical flasks according to size, containing a small volume of water, usually 5 ml. This retained the nitrogenous excreta, diluted them adequately and permitted adequate oxygenation.

Little NH_3 appeared to be lost to the air from this medium or even from a minimal volume of water, just sufficient to keep the body surface wet. When a stream of ammonia-free air was bubbled through the medium continuously, and then passed through standard H_2SO_4 after being freed of CO_2, an average of 3·5% of the ammonia excreted was carried over. This may not be significantly greater than the experimental error, for the same absolute amount was carried over from a minimal volume of medium, though the percentage was higher because this condition depresses NH_3 output (p. 438). Under the standard laboratory conditions, with still air, and 5–10 ml. of water in a lightly stoppered flask, the loss was certainly insignificant. Worms survived for periods up to 6 months in this condition without evident ill-effects.

The flasks were kept in the dark, in a water-bath regulating at $23 \pm \frac{1}{2}$° C.; this temperature avoided the need for a cooling system at any time, and appeared to cause the worms no distress. *Eisenia*, living in compost and manure heaps, is probably accustomed to high temperatures, but the normal temperature (and rate of metabolism) of *Lumbricus* is no doubt lower than this.

The medium was a mixture of equal parts of tap water and distilled water, and was renewed daily at the same hour. Its content of the relevant nitrogen components was estimated immediately afterwards. The amino plus ammonia (AA) fraction and this fraction plus urea (AAU) have been regularly estimated, and total nitrogen in a representative number of cases. A limited number of estimates were made of uric acid, of protein and non-protein fractions and of the percentage of NH_3 in the AA fraction. The AA fraction was estimated by the Sörensen formol method and the AAU fraction by the same method, after conversion of the urea to NH_3, with 5 mg. of soya-urease per sample. Total nitrogen (KN) was estimated by the Kjeldahl method, using a Markham still for the steam distillation. Protein nitrogen was precipated by zinc sulphate in alkaline medium, and both protein and non-protein fractions then estimated by the Kjeldahl method. Ammonia alone was measured by direct steam distillation of the medium, made alkaline after introduction into the still. In *Lumbricus* virtually 100% of the AA fraction was NH_3 and for that genus AA will usually be referred to as NH_3 but in fasting *Eisenia*, only 86% was NH_3 and in feeding *Eisenia* still less (67%).

The nitrogen measured was that excreted via all pathways, and in feeding animals it included possible traces of faecal nitrogen. It is difficult to devise a method of excluding all faeces from the excretory medium, which would be practicable for relatively large numbers of individually isolated worms, and so no separation was attempted. Experiment showed that there was no measurable quantity of nitrogen in the solid faecal matter, but there may have been some soluble faecal nitrogen.

Well-steeped elm leaves, of the leaf-fall, were used as food. This material has the merit of releasing virtually no soluble nitrogen into the water and therefore permits feeding under the present experimental conditions. It contains 6·6 mg. of nitrogen per gramme dry weight. The food is perhaps not entirely adequate at this temperature, and even feeding worms, of both species, often lost some weight, but it gave an adequate contrast between feeding and fasting conditions and between the responses to these. For some purposes filter paper was used, as a nitrogen-free food, and was readily accepted, especially by *Lumbricus*.

In all, 180 worms were studied, each through a series of changes of conditions. Complete equilibration to changed conditions often requires a month or more (Cohen & Lewis, 1949*b*), and therefore no attempt has been made to ensure complete equilibration, and the interest is essentially in the initial, major responses to new conditions. All experiments were 'staggered' for the different individuals, often by long periods, so that any possible fortuitous variables were asynchronized.

Burian has objected that the nephridia evacuate at intervals longer than 24 hr. (Delaunay, 1931), and in the present records the output of some individuals at certain times oscillated from day to day to a degree consistent with this contention, but such cases were not so common as to obscure the significant responses in the population as a whole. There was no good evidence that all the nephridia usually evacuated synchronously, at intervals greater than 24 hr.

For brevity, the expressions 'AA output', etc., will be used to mean 'AA nitrogen output', etc.

RESULTS

A. Lumbricus terrestris

The average 'specific' values of AA, AAU and KN outputs (p. 426) in μg. of nitrogen per gramme of body weight per day, obtained from all available records, are given in Table 1. In fasting *Lumbricus* the mean specific value of KN was 331 μg./g./day which represents a loss of between 1·09 and 1·65 % of the body weight per day, assuming that nitrogen accounts for 2–3 % of the body weight (Lesser, 1908). Direct measurements of the weight loss for worms on a varied régime gave a value of 1·3 % per day, compound interest, but for worms fasting for long periods the value would be greater than this and a loss also of non-nitrogenous material has been demonstrated (Lesser, 1908). Direct measurements of weight loss over short periods of fasting or feeding are complicated by the considerable changes in weight of the gut contents, and few records were taken of worms fasting for long periods. These indicated weight losses as high as 2·4 %/day.

Table 1. *Specific mean output (μg.) per day of the major fractions of nitrogen excreted by* Lumbricus *and* Eisenia

Condition	AA	Urea (by difference)	AAU	Residual N (by difference)	KN
Lumbricus					
Fasting	30·1 (755)	129·1	159·2 (1612)	172·2	331·4 (27)
Feeding on filter paper	29·5 (155)	126·0	155·5 (320)	160·9	316·4 (77)
Feeding on elm leaf	54·0 (705)	40·8	94·8 (940)	174·0	268·8 (105)
Isolated posterior pieces	56·4 (165)	52·1	108·5 (687)	352·7	460·2 (68)
Eisenia					
Fasting	272·4 (841)	73·1	345·5 (1898)	223·5	569·0 (54)
Feeding on filter paper	119·6 (218)	50·2	169·8 (291)	148·1	318·9 (9)
Feeding on elm leaf	156·5 (1670)	48·3	204·8 (2515)	197·0	401·8 (294)
Isolated posterior pieces	164·6 (44)	87·8	252·4 (400)	432·5	684·9 (33)

In this, and in Tables 2–5 all values are μg. of nitrogen per g. wet weight per day. AA = amino nitrogen + ammonia nitrogen; AAU = AA + urea nitrogen; KN = total nitrogen; residual = KN − AAU.

In this table the figures in parentheses are the numbers of records on which the mean values are based.

The mean specific output of KN by worms feeding on leaf was 269 μg. i.e. less than that of fasting worms; this indicates some sparing of body protein by the food, particularly as the figure may include some unassimilated food nitrogen. The maximal consumption was 80 mg. dry weight of leaf per day, i.e. about 0·5 mg. of nitrogen (p. 426), the average being about one-third of this, so that the food nitrogen was sometimes adequate for positive nitrogen balance. This perhaps is indicated also by a progressive increase in KN which was observed in worms feeding over a protracted period, as though body nitrogen was progressively approaching saturation, but in fact this increase may have been one manifestation of a progressively decreasing intake due to an inadequacy of the diet (p. 426). For those *Lumbricus* feeding on filter paper, the mean specific value of KN was 316μg./day, only slightly less than for fasting worms. There was no evidence of extensive digestion of this material.

The mean specific AAU output of fasting *Lumbricus* was 159μg./day, 48·0% of the total nitrogen. The nature of the remainder (= 'residual nitrogen') was not investigated, but it was quantitatively, and probably also qualitatively, very similar to that of feeding worms of the species (p. 430), i.e. largely protein, probably mucus. In feeding worms specific AAU output was absolutely (Table 1), and also relatively, lower than in fasting individuals, being 35·3% of the total nitrogen. Feeding on leaf therefore 'spares' this fraction. For worms feeding on filter paper, specific AAU output was slightly, but not much, lower than in fasting animals. The residual nitrogen was approximately the same as in the other two groups.

The mean specific output of ammonia (virtually the whole of the AA fraction, p. 426) was 9·1% of KN in fasting worms and 9·3% in those feeding on filter paper, but considerably greater (20·1%) in those feeding on elm leaf. This value includes records for transitional periods from fasting (and for individuals with indifferent appetites), and the percentage in fact was sometimes as high as 27%, or about three-quarters of the AAU fraction. Similarly, the mean value here given for fasting worms is much higher than in individuals fasting for periods greater than a week or so, when it declined to an insignificant amount (Fig. 2), as Lesser (1908) observed. The amount of urea excreted varied approximately in inverse proportion to the difference in NH_3 output between fasting and feeding worms. In fasting worms the mean NH_3/urea ratio was 0·23 compared with 1·32 when feeding. When feeding on filter paper the value was virtually the same as when fasting.

Lesser (1908), Delaunay (1934) and Cohen & Lewis (1949, 1950) observed a striking decline in NH_3 output and a corresponding increase in urea output by worms fresh from the field, and their evidence has increasingly established that this was due to the onset of fasting. This was again illustrated by worms fasting at this time in the present experiments (Fig. 1), whereas a control group, feeding throughout, showed a very limited change in pattern of output. In the feeding group NH_3 output increased slightly. From Fig. 1 it is seen that the change in urea output in fasting worms was much greater and more prompt than that in NH_3 output, which further strengthens the evidence of Cohen & Lewis (1949) that the ammonia found in the medium is not produced from urea after liberation from the body.

The main response to diet appeared to begin around the third or fourth day in the laboratory, the initial period presumably involving adjustment to laboratory conditions generally (p. 438). The maximal output of urea by fasting worms was reached on the tenth day, after which *both* NH_3 and urea outputs declined somewhat. In feeding worms both increased somewhat so that a reciprocal change in NH_3 and urea is not invariable.

Records during transitions between feeding and fasting, in worms already acclimatized to laboratory conditions (Fig. 2), further confirmed the relationship between the NH_3/urea ratio and feeding. Again urea output changed more rapidly and extensively than NH_3 output, at the transition, irrespective of its direction.

AAU output therefore showed a considerable change, particularly during the first few days, in the same direction as the urea output. In fasting worms AA output fell virtually to zero in 7–10 days.

During a prolonged sojourn in the laboratory there was a progressive fall in the output of NH_3 and a rise in that of urea and KN, in both fasting and feeding *Lumbricus*, i.e. a trend in the general direction of the fasting pattern of output. This is probably evidence of inadequacies in the diet (p. 426), or possibly in other laboratory conditions.

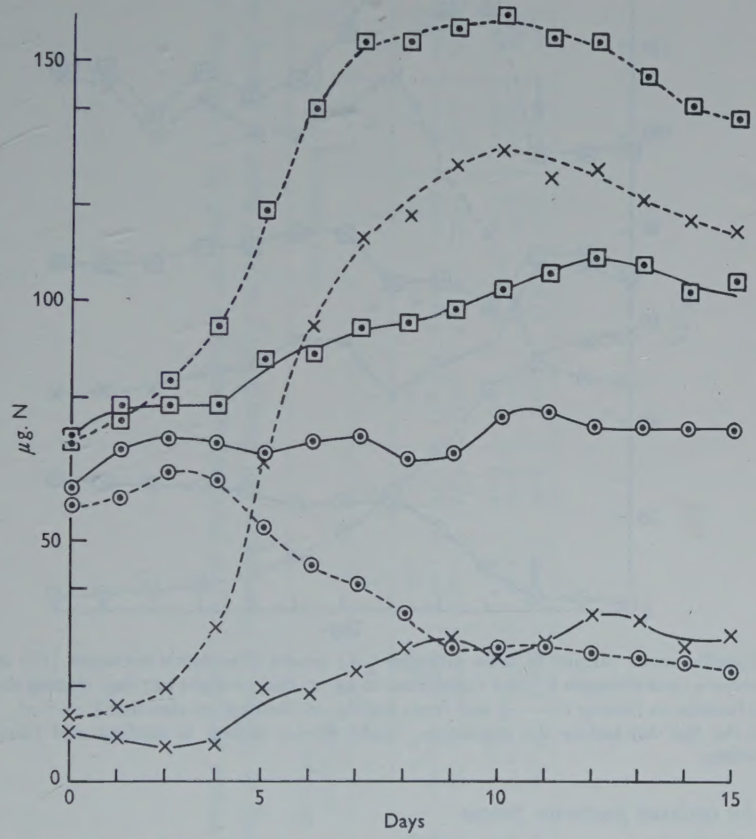

Fig. 1. Specific mean output of urea nitrogen (×), ammonia + amino nitrogen (⊙), and ammonia + amino + urea nitrogen (⊡) by *Lumbricus*, in μg/g. body weight per day, during the first 15 days in the laboratory, (– – – –) when fasting and (————) when feeding on elm leaf. Twenty-seven fasting and fifteen feeding worms.

The transition from fasting, via a diet of filter paper, to one of leaves (Table 2) showed that filter paper in fact does cause a small increase in NH_3 output over that of fasting worms and a correspondingly larger decline in urea output. The change was qualitatively the same as that due to leaf and a more easily assimilated carbohydrate might have an effect which was quantitatively comparable also. The changes due to filter paper were nearly a half of those due to the further transition to feeding on leaf.

A limited number of estimates, on feeding worms, indicated that uric acid accounted for 1·5 % of the excreted nitrogen. Estimations of the total non-protein nitrogen indicated that this was 51·7 % of the total nitrogen, that as much as 16·4 % of the total was in forms other than NH_3 and urea (which together constituted 35·3 % (p. 428)), and protein (48·3 %).

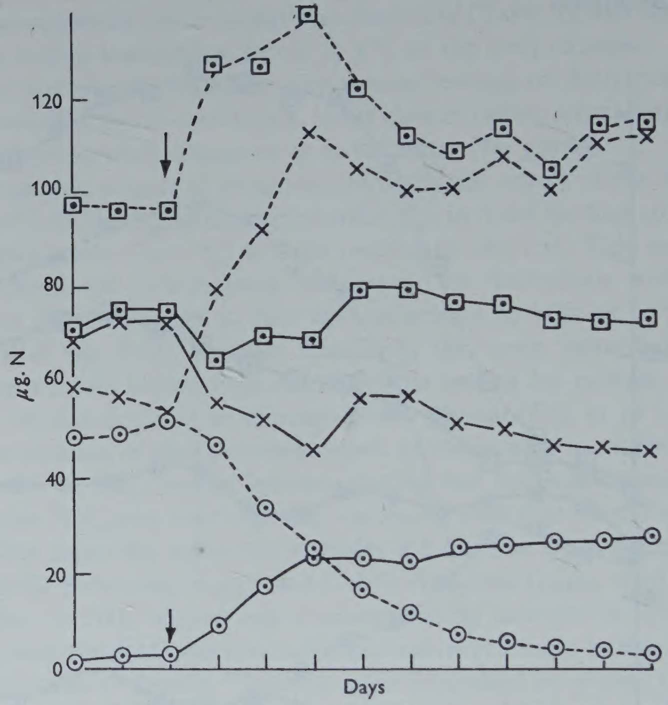

Fig. 2. Specific mean output of urea nitrogen (×) amino + ammonia-nitrogen (⊙) and amino + ammonia + urea nitrogen (⊡) by *Lumbricus*, in µg./g. body weight per day, during the transitions from feeding to fasting (----) and from fasting to feeding on elm leaf (———). The arrows mark the last day before the transition. Eight worms fasting to feeding and thirteen feeding to fasting.

Output by isolated posterior pieces

Such isolated pieces are potentially interesting for the light they might throw on: (i) intrinsic differences in the tissue along the length of the body, (ii) the effects of isolation from the whole worm, and (iii) the effects of wound healing. In fact they did not regenerate and their healing was often imperfect but they often lived for several weeks. Their specific outputs of NH_3 and urea were virtually identical with those of whole worms feeding on leaf (Table 1), though in fact these pieces are unable to feed, and were never given food after the inability was demonstrated. The mean value of KN (Table 1) was based on records from a limited number of pieces, but is probably reliable since the output of isolated pieces is relatively steady. It indicates a relatively high value of KN and of residual nitrogen, i.e. probably mucus.

Table 2. *Specific mean output per day (μg.) of fractions of nitrogen excreted by* Lumbricus *during the transitions (a) from fasting to feeding on filter paper, (b) from feeding on filter paper to feeding on elm leaf*

Transition and fraction	No. of records	Days prior to transition			Days following transition									
		3	2	1	1	2	3	4	5	6	7	8	9	10
(*a*) AA	4	4·3	3·2	2·7	6·0	7·7	8·9	10·0	9·7	8·4	10·7	14·6	14·0	12·3
AAU	11	143·4	141·0	137·1	136·1	133·1	128·5	125·7	119·6	122·1	125·3	120·4	133·3	118·4
U	By difference	139·1	137·8	134·4	130·1	125·4	119·6	115·7	109·9	113·7	114·6	105·8	119·3	106·1
(*b*) AA	4	15·7	13·6	11·9	19·2	21·9	30·1	38·0	42·3	46·9	45·1	42·9	35·1	27·6
AAU	8	118·7	121·0	123·1	76·7	81·8	92·8	95·9	95·6	88·6	87·8	89·9	83·2	80·5
U	By difference	103·0	107·4	111·2	57·5	59·9	62·7	57·9	53·3	41·7	42·7	47·0	48·1	52·9

B. Eisenia foetida

This worm, the brandling, is smaller than *Lumbricus terrestris*. Specimens used ranged from 200 to 600 mg. wet weight, not much more than one-tenth of the average size of the *Lumbricus*. They were obtained locally from heaps of leaf-mould. The genus is known to differ from *Lumbricus* in a number of respects (Stephenson, 1930), but even so the differences in pattern of nitrogen output which have been observed are surprisingly great, often diametrically contrasting.

The total nitrogen output per gramme per day was considerably greater (Table 1), though as in *Lumbricus* it was greater in fasting than in feeding animals. This difference itself was greater, both absolutely and relatively, than in *Lumbricus*. For *Eisenia* feeding on filter paper, the mean of available values for specific KN output was lower than for leaf-feeding individuals and this may be another real contrast (p. 428) to the condition in *Lumbricus*; the figures were based on few records, but those for AA and AAU were more adequate and show the same feature (Table 1). Certainly the pattern of output was much nearer to that of leaf-fed than to that of fasting worms, in contrast to the pattern in *Lumbricus*, and filter paper would seem to spare body protein more effectively than in *Lumbricus*. For *Eisenia* it is possibly more effective than elm leaf.

The specific AAU output of fasting *Eisenia* was 60·7% of KN, a higher percentage of the total than in *Lumbricus*, and more than twice as great in absolute value. In absolute magnitude and as a percentage of KN (51·0%), AAU was lower in feeding than in fasting *Eisenia*—this difference being qualitatively the same as in *Lumbricus*, and absolutely greater, though relatively smaller. When feeding on filter paper AAU output, like KN output, was lower than when feeding on leaf.

The greatest contrasts to the pattern in *Lumbricus* were (i) the absolutely and relatively high value of specific AA output and (ii) the fact that AA output was not lower but higher in fasting than in feeding animals, viz. 47·8% compared with 38·9% of the total—about the same as the respective values of AAU in *Lumbricus*. Specific urea output, on the other hand, was lower than in *Lumbricus* and only about 12% of the total. It was higher in fasting than in feeding worms, however, as in *Lumbricus*. Since the AA fraction contained 14 and 33% respectively, in fasting and feeding *Eisenia* (p. 426), of nitrogen other than that of NH_3, the absolute amounts, and percentages, of NH_3-nitrogen were lower than the figures given above, viz. 41·4 and 26·1% of KN respectively. Even so, the specific NH_3-output was much higher than in *Lumbricus* and the feeding/fasting difference was more pronounced than in the case of the whole AA fraction. Although in fasting worms NH_3 was a much higher percentage of the total N than in *Lumbricus* the percentage for feeding worms was not very different in the two species.

These peculiarities of *Eisenia* were confirmed by a comparison of feeding and fasting individuals during the first fortnight after entry to the laboratory (Fig. 3) In fasting worms there was an immediate large increase in AA output on the first day, after which it declined almost to the initial level for feeding individuals on the fourth and fifth days and then rose somewhat to a lower peak on the tenth day. The times of the main turning points on the *Eisenia* curves correspond rather closely with those for *Lumbricus* (Fig. 1), although the two species differ so markedly in the nature of the changes. The AA values for feeding *Eisenia* remained throughout lower than those for fasting individuals. Urea output was at first lower than that of feeding worms, but eventually rose somewhat above the latter which declined irregularly throughout. In fasting worms there was a slight long-term decline in AA ouput, and an increase in urea output, which conceivably may represent a vestige of what is the main trend in *Lumbricus* (p. 428). Apart from minor oscillations AA output remained constant in feeding worms. The initial downward trend during the first 5 days (p. 428) was in the same direction in both AA and urea and in both fasting and feeding worms. *Lumbricus* showed an almost equally uniform, but smaller, *increase* in all fractions (Fig. 1). The significance of this species difference, and of the minor oscillations of the record in general, probably merit further study. It seems probable that in general they are significant. The sharp and temporary increase in AA output in feeding *Eisenia* which occurs between days 3 and 5 was chosen for test because of its surprising incongruence and was found to be significant at almost the 1% level ($t=2\cdot52$, $n=29$). An indication of this increase in fact is seen also in the independently estimated AAU values (Fig. 3). Some of the oscillations here, and in other figures and tables, may be of the type described in *Carcinus* (Needham, 1957), but this particular change seems more peculiar. In

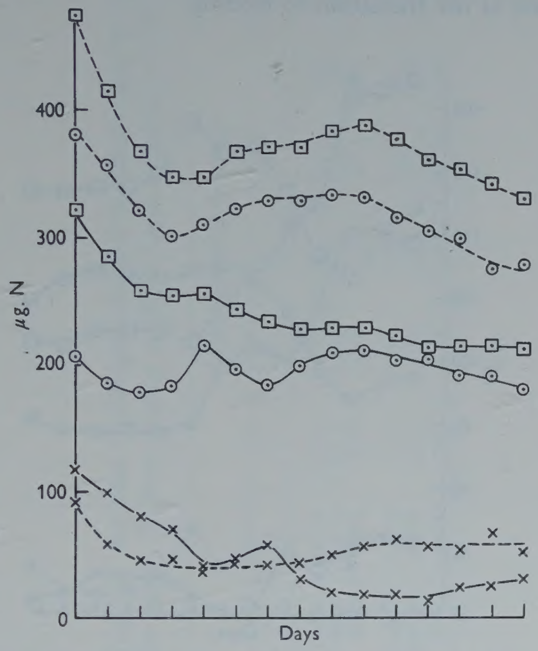

Fig. 3. Specific mean output of urea nitrogen (×), amino+ammonia nitrogen (⊙) and amino+ammonia+urea nitrogen (⊡) by *Eisenia*, in µg./g. body weight per day, during the first 15 days in the laboratory (----) when fasting and (———) when feeding. Twenty-nine fasting and thirty feeding worms.

feeding *Eisenia*, particularly, AA and urea outputs showed reciprocal fluctuations which are even more pronounced in certain related curves (Fig. 6).

That AA output is considerably higher in fasting than in feeding *Eisenia* was confirmed by estimations made during the transitions, both ways, between fasting and feeding, after acclimatization to laboratory conditions (Fig. 4). Urea output had become low and remained relatively constant apart from minor and brief fluctuations, but AA output climbed sharply at the transition to fasting and declined even more at the transition to feeding.

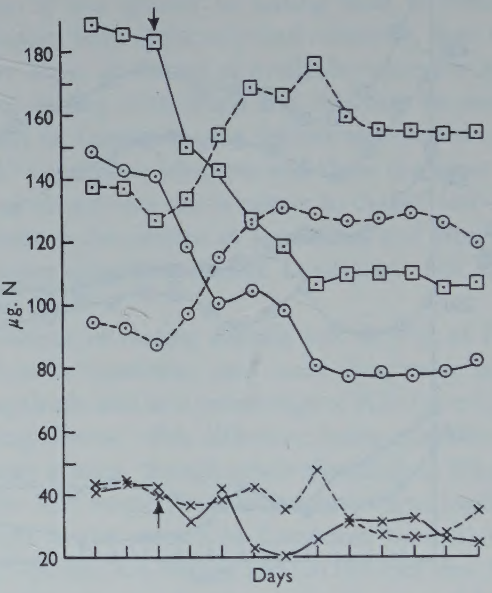

Fig. 4. Specific mean output of urea nitrogen (×), amino+ammonia nitrogen (⊙) and amino+ammonia+urea nitrogen (⊡), by *Eisenia*, in μg./g. body weight per day, during the transitions from feeding to fasting (----), and from fasting to feeding on elm leaf (———). The arrows mark the last day before the transition. Thirty-two worms fasting to feeding and thirty-nine feeding to fasting.

At the transition from fasting to feeding on filter paper *Eisenia* showed a marked fall in AA output (Table 3), and at the further transition to feeding on leaf there was, after a small increase, a further but much smaller fall. This is in agreement with the evidence on Table 1 that for *Eisenia* filter paper is more nearly equivalent to leaf in food value than for *Lumbricus* (p. 432). Urea output possibly declined somewhat but again showed no large or sustained change, though it oscillated rather violently and these large oscillations may prove to have some significance. AA output showed much smaller and slower fluctuations. The oscillations in urea output followed much the same pattern at each of the two transitions; there were two complete cycles of increase and decrease, with a period of about 5 days. It is surprising, perhaps, that the oscillations were not equally great after the direct transition from fasting to feeding on leaf (Fig. 4).

Table 3. *Specific mean output per day (μg.) of fractions of nitrogen excreted by* Eisenia *during the transitions (a) from fasting to feeding on filter paper, (b) from feeding on filter paper to feeding on elm leaf*

Transition and fraction	No. of records	Days prior to transition			Days following transition									
		3	2	1	1	2	3	4	5	6	7	8	9	10
(*a*) AA	18	138·3	145·7	146·4	134·3	121·4	110·2	104·6	100·8	93·1	79·7	75·9	78·4	84·3
AAU	25	199·4	185·9	191·7	192·9	190·0	167·2	134·4	132·2	121·1	128·2	141·4	128·8	123·5
U	By difference	61·1	40·2	45·3	58·6	68·6	57·0	29·8	31·4	28·0	48·5	65·5	50·3	39·2
(*b*) AA	17	96·9	89·9	95·8	105·6	105·8	97·7	88·3	83·9	79·7	77·4	83·0	81·9	76·0
AAU	24	167·9	140·1	142·5	167·2	148·8	131·3	130·2	141·5	147·0	148·3	130·8	127·7	127·4
U	By difference	71·0	50·2	46·7	61·6	43·0	33·6	41·9	57·6	67·3	70·9	47·8	45·8	51·4

Although these results indicate that AA output and urea output were somewhat lower when feeding on leaf than on filter paper, contrary to the evidence of Table 1, it is noteworthy that both increased, if only temporarily, after the transition from feeding on filter paper to leaf.

During a prolonged sojourn in the laboratory AA output declined, and urea output and KN output rose; this was observed in all, but particularly in fasting, *Eisenia*. In these respects *Eisenia* resembles *Lumbricus*.

A limited number of estimates of uric acid output in *Eisenia* gave values of 3·40 and 3·35% respectively of the total nitrogen for fasting and feeding worms. Estimations of total non-protein nitrogen indicated that AAU accounted for 95·5% of that fraction so that there was probably less nitrogen unaccounted for than in *Lumbricus*, and virtually all of the 'residual nitrogen' (p. 428) was protein.

Isolated posterior pieces of Eisenia.

As in *Lumbricus* the specific output of KN by isolated posterior pieces was higher than for the intact worm (Table 1) and this was again due to residual nitrogen, mainly protein. AA output and AAU output were intermediate between those of fasting and feeding worms, and as in *Lumbricus*, much nearer those of the feeding than of the fasting condition, notwithstanding the radical differences between the patterns of output characteristic of *Lumbricus* and of *Eisenia*.

C. *The effect of variations in the volume of the medium*

There were three main reasons for investigating the effect of this variable on the pattern of nitrogen output of these worms: (i) earthworms are probably not normally immersed in water for long periods, and (ii) the volume of the medium has sometimes been suspected of influencing the pattern, particularly the NH_3/urea ratio (Prosser, 1950, p. 197), (iii) the nitrogen output of some aquatic animals is very sensitive to changes in volume of the medium (Needham, 1957).

The effects here were smaller than might have been anticipated (Tables 4 and 5). They were also usually transitory, probably because any restriction of output, or any 'washing out' of nitrogen, automatically leads to the compensatory trend in due course (Needham, 1957). The resulting oscillations were often still evident in the days immediately before the next transition (Tables 4 and 5).

When feeding *Lumbricus* were transferred from 10 to 5 ml. of medium (Table 4) there was some increase in AA output above the general level prior to the transition, to which level it returned after about a week. The output of urea increased somewhat and remained generally higher than before the transition. The reciprocal transition was followed by a more definite and sustained decrease in AA output and also by an equally pronounced initial increase in urea output. It is probably legitimate, therefore, to suggest that the increase in volume of the medium had a slight effect on output in the same direction as that due to fasting and that a decrease in volume had the opposite effect. There was some further evidence for this from *Eisenia*, in which fasting causes an increase in AA output (p. 432). When the volume of the medium was increased in fact there was little change in AA output (Table 5),

Table 4. *Specific mean output (μg.) per day of fractions of nitrogen excreted by* Lumbricus *during the transitions (a) from 5 to 10 ml. of medium, (b) from 10 to 5 ml. of medium, (i) when fasting, (ii) when feeding on elm leaf*

Transition and fraction	No. of records	Days prior to transition			Days following transition									
		1	2	3	1	2	3	4	5	6	7	8	9	10
(*a*) (ii) AA	12	56·6	58·5	58·7	57·5	55·7	51·0	50·4	50·1	48·9	49·6	47·9	48·0	48·9
AAU	12	91·1	87·2	90·0	99·8	97·3	93·5	95·3	90·4	69·2	70·1	74·8	79·2	79·9
Urea	By difference	34·5	28·7	31·3	42·3	41·6	42·5	44·9	40·3	20·3	20·5	26·9	31·2	31·0
(*b*) (i) AAU	7	203·0	228·9	269·8	273·8	286·2	231·1	222·0	273·8	288·0	240·5	241·1	248·1	232·1
(ii) AA	11	46·6	46·9	55·7	56·1	54·5	53·6	54·5	57·0	53·8	47·0	51·8	49·4	47·9
AAU	11	70·1	75·9	81·2	86·1	90·6	83·6	81·3	84·0	85·5	82·6	82·3	79·4	83·9
Urea	By difference	23·5	29·0	25·5	30·0	36·1	30·0	26·9	27·0	31·8	35·6	30·5	30·0	36·0

Table 5. *Specific mean output (μg.) per day of fractions of nitrogen excreted by* Eisenia *during the transitions (a) from 2 to 5 ml. and (b) from 5 to 2 ml. of medium, (i) fasting and (ii) when feeding on elm leaf*

Transition and fraction	No. of records	Days prior to transition			Days following transition									
		3	2	1	1	2	3	4	5	6	7	8	9	10
(*a*) (i) AA	10	441·1	387·7	416·4	432·5	387·7	364·7	402·9	411·5	419·2	434·4	407·7	375·2	382·9
AAU	10	505·8	488·7	492·4	444·8	412·6	437·6	438·6	490·6	524·7	507·6	496·0	476·3	407·8
Urea	By difference	64·7	101·0	76·0	12·3	24·9	72·9	35·7	79·1	105·5	73·2	88·3	101·1	87·9
(ii) AA	18	94·6	87·6	80·1	83·7	88·6	74·1	69·7	77·7	79·4	75·0	77·0	75·0	78·8
AAU	18	112·1	121·2	120·3	119·8	126·7	142·4	143·9	139·4	136·5	113·1	93·2	92·0	89·0
Urea	By difference	17·5	33·6	40·2	36·1	38·1	68·3	74·2	61·7	57·1	38·1	16·8	17·0	10·2
(*b*) (i) AA	10	390·2	411·6	383·9	356·0	366·8	382·8	347·1	304·9	299·6	301·3	—	—	—
AAU	10	525·6	504·0	483·4	471·8	477·1	470·8	439·5	452·1	464·5	415·2	403·6	414·4	418·9
Urea	By difference	135·4	92·4	99·5	115·8	110·3	88·0	92·4	147·2	164·9	113·9	—	—	—
(ii) AA	23	72·9	73·1	67·2	69·2	65·9	60·5	56·4	53·5	67·5	71·4	61·5	56·0	54·5
AAU	23	116·9	99·1	93·2	93·9	100·4	100·0	96·9	94·5	91·0	92·0	88·1	93·9	96·9
Urea	By difference	44·0	26·0	26·0	24·7	34·5	39·5	40·5	41·0	23·5	20·6	26·6	37·9	42·4

possibly a slight increase; but when the volume was decreased there was a definite and sustained decrease in AA output, in both fasting and feeding worms of this species.

The results for *Lumbricus*, if significant, are in the reverse direction to that postulated (Prosser *et al.* 1950) for changes in volume of the medium, but there was some evidence from the present experiments that a drastic reduction in volume—from 5 ml. to the minimum (p. 425)—had the same effect on output as an increase from 5 to 10 ml., and therefore in the direction postulated. Certainly there was an immediate decrease of 20–40% in AA output on the first day and the pattern of output by worms kept continuously in a minimal volume of medium was an exaggeration of the fasting pattern. For a group of seven worms, fasting in minimal volume during the first fortnight in the laboratory, the average specific outputs of AA and urea were 37·5 and 106·8μg. compared with 41·3 and 85·7μg. respectively for the fasting worms illustrated in Fig. 1.

Eisenia showed the same response to minimal volume, after an initial fall in output on the first day, viz. a pattern of output resembling the fasting condition. For a group of eleven feeding individuals, in minimal volume during the first fortnight in the laboratory, the average specific values of the AA output and urea output were 304·8 and 51·9μg. compared with 196·0 and 45·5μg. respectively for the feeding worms illustrated in Fig. 3. The decline in both AA output and urea output during the first 5 days in water in the laboratory, by both fasting and feeding *Eisenia* (Fig. 3) is probably to be regarded as the reciprocal of this effect. During the same period the output of *Lumbricus* likewise (Fig. 1) showed the reciprocal response to that when in a minimal volume of water, viz. an increase in AA output and a decrease in urea output—the latter masked in fasting worms by the much larger response to fasting (p. 428).

The volume of the medium therefore probably has some small influence on the pattern of nitrogen output, particularly immediately after a sudden change in volume. There is possibly an optimal volume, around 2 ml. per worm for *Eisenia* and around 5 ml. for *Lumbricus*. Changes in either direction from this have the same effect, which resembles that due to fasting, and is therefore non-specific and possibly a 'stress' effect (Needham, 1957). *Eisenia* and *Lumbricus* maintain their species-specific differences in these responses to changes in volume of medium.

The effect of frequency of renewal of medium

To some extent the available volume of medium is affected by the frequency of its renewal. A number of worms of both species were kept each in 5 ml. of water, unrenewed, for 14 days. Most of these survived and the total AAU output of fourteen *Eisenia* during the period was 2009μg./g. body weight, compared with 165μg. for the last day before this period. For three *Lumbricus* the values were 1168 and 86μg. respectively. In both species therefore the output was very closely proportional to the time of exposure to the medium. During this time the animals were fasting but had been feeding immediately previously, so that unchanged medium in fact may depress output somewhat, i.e. approximately to the level of that of feeding individuals. Worms may re-consume faeces under these conditions and so approximate to the feeding condition. In any event the very small depression of output contrasts with the marked effect of stagnant medium on that of *Carcinus* (Needham, 1957), and it is therefore not surprising that these worms are relatively insensitive also to small changes in the volume of the medium (p. 436).

That there is some depression of output, however, was shown by estimations on eight isolated posterior pieces, which therefore had been fasting for some time before the period of unrenewed medium. The mean output of AAU was 59·5 μg./g. on the last day before this period, 44·8 μg./day during the period, and 71·4 μg. on the first day after return to daily renewal. There was thus on the average a 25 % depression of output, and an approximately equal excess over normal, i.e. a washing out of accumulated nitrogen, after return to the daily routine. A limited number of observations on fasting intact *Lumbricus* were in accord with this result.

There was also evidence that an unrenewed medium caused serious stress on the animals, since of the seventeen worms which were exposed to 14 days of this treatment eight died between the sixth and eighth days after return to normal conditions. The remainder lived indefinitely, i.e. there appeared to be sharply defined critical period for this stress-response.

D. Allolobophora caliginosa

This species resembled *Lumbricus* in most of the features which are relevant here. Thus KN output and urea output increased and NH_3 output decreased at the onset of fasting. The worm appeared to excrete considerably less per unit weight than *Lumbricus*: its specific outputs of AA, AAU and KN averaged 14·0, 97·2 and 133·3 μg./g./day respectively when fasting and 37·4, 57·3 and 87·5 μg. when feeding. It therefore appears to have a more economical metabolism than *Lumbricus*. Possibly for this reason its appetite was relatively poor and feeding sometimes sporadic.

Isolated posterior pieces showed a specific daily output of 11·6 μg. of AA and 100·1 μg. of AAU, values which are very near those for *fasting*, and not to those of feeding worms as in *Lumbricus* and *Eisenia* (pp. 430, 436). The pieces of this worm were more viable than those of *Lumbricus* and *Eisenia* and survived indefinitely at room temperature, though their powers of regeneration were not conspicuously better than those of posterior pieces of the other two species. *Allolobophora caliginosa* has a much firmer body than the other two species, and evidently differs in a number of physiological features.

DISCUSSION

The present results confirm previous evidence (Lesser, 1908; Delaunay, 1931, 1934; Cohen & Lewis, 1949*a*, *b*) that fasting causes an increase in the urea/NH_3-output ratio in *Lumbricus*, and show that re-feeding reverses the ratio once more. They also support the evidence of Cohen & Lewis (1949*b*) that the increase in NH_3 when feeding is not due to its formation from excreted urea by gut bacteria, since the change in urea output is much greater than that of NH_3, and the two changes have different time courses (pp. 428–9). Moreover, *Eisenia* produces more NH_3 when fasting than when feeding. The results best support the view of Delaunay (1931) that the changes are part of a positive buffering mechanism of the animal, a view supported by experiments on leeches (see Delaunay, 1934).

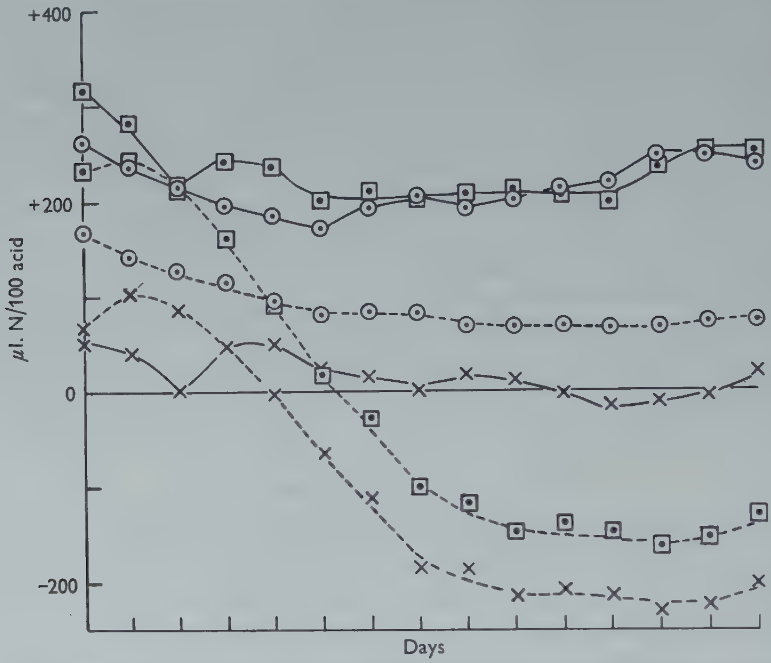

Fig. 5. Titratable acid (phenolphthalein as indicator) in μl. N/100 acid per g. body weight per day, produced by *Lumbricus* during the first 15 days in the laboratory, (– – – –) when fasting and (———) when feeding on elm leaf, (⊙) as estimated on the freshly withdrawn medium, (⊡) as estimated after conversion of all urea to ammonia, (×) difference between the other two curves. Media alkaline to the indicator are recorded as containing negative quantities of titratable acid. Twenty-seven fasting and fifteen feeding worms.

In the present records feeding *Lumbricus* passed more acid into the medium than fasting worms (Fig. 5)—as Darwin first anticipated (Robertson, 1936). Acid-production has been measured by the amount of NaOH required to neutralize the medium to phenolphthalein (pH 8·3), as a preliminary to Sörensen estimations (p. 426). This permits the estimation of acid already neutralized to pH 7 by the worms. The 'titratable acid' was consistently higher from feeding than from fasting *Lumbricus* (Fig. 5), and this difference was confirmed by records (Table 6) during the feeding fasting transitions (p. 428). Treatment of the medium from fasting worms with urease caused a marked increase in alkalinity due to the NH_3 released (Fig. 5), the pH rising above 8·3 (acidity therefore recorded as a negative quantity, equal to the back-titration). This sufficiently emphasizes the physiological importance of the normal increase in urea/NH_3 output by fasting worms.

Assuming that this buffering mechanism operates also in *Eisenia*, then its high NH_3 output when feeding and even higher output when fasting (Table 1) might indicate (1) that it produces much more acid than *Lumbricus* per unit weight, and

Table 6. *Titratable acid produced per g./day by* Lumbricus *and* Eisenia *during the transitions (a) from fasting to feeding, and (b) from feeding to fasting (i) in the medium as initially separated, and (ii) after conversion of any urea present to ammonia*

Transition and condition	No. of records	Days prior to transition			Days following transition									
		3	2	1	1	2	3	4	5	6	7	8	9	10
Lumbricus														
(*a*) (i)	6	38	42	44	94	136	143	149	157	155	163	161	173	167
(ii)	6	0·07	−38	−44	90	161	219	232	235	264	272	237	223	224
(*b*) (i)	8	168	176	182	131	77	65	62	49	38	35	33	35	38
(ii)	8	225	204	184	72	22	−10	+21	+17	−31	−48	−68	−87	−94
Eisenia														
(*a*) (i)	25	535	476	476	774	844	1102	1258	1349	1387	1475	1498	1522	1606
(ii)	25	808	735	724	1007	1247	1463	1450	1414	1566	1716	1837	1917	1710
(*b*) (i)	28	1059	1159	1160	880	613	612	650	597	551	553	504	435	422
(ii)	28	1471	1378	1223	916	831	836	794	695	650	632	630	665	671

The figures are mean values in μl. of N/100 acid produced per g wet weight per day.
The indicator was phenolphthalein and the 'acid' measured had always been neutralized by the animal to approximately pH 7·0 (see text).

(2) that it produces more when fasting than feeding. The first supposition proved correct (Fig. 6) but not the second. On the contrary, *Eisenia* resembled *Lumbricus* in producing more acid when feeding; this effect was even more pronounced in *Eisenia*. The simplest conclusion is that *Eisenia* does make use of the *Lumbricus* method of buffering, since its specific output of acid is greater than that of *Lumbricus* by a factor comparable to that relating their specific AA outputs, but that it also uses a second mechanism when feeding. The total nitrogen output decreased when feeding (Table 1) so that there may be insufficient available as NH_3 to buffer the extra acid. Some of the acid produced by feeding but not by fasting *Eisenia* appeared to ionize slowly, i.e. it delayed the end-point with phenolphthalein.

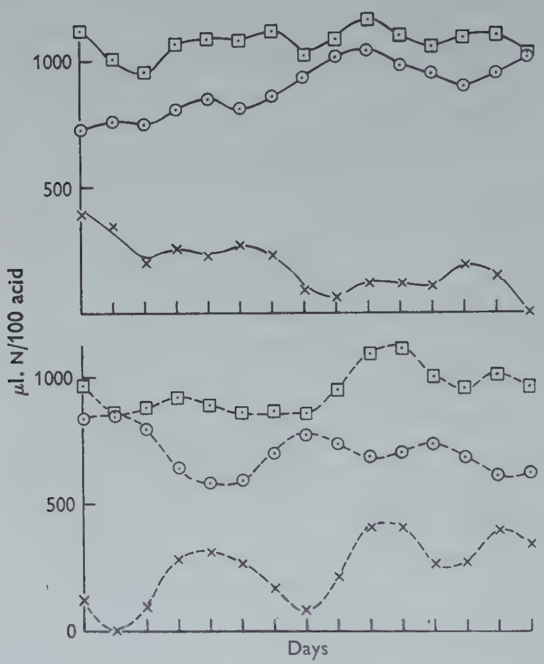

Fig. 6. *Eisenia*. Legend otherwise as in Fig. 5. Twenty-nine fasting and thirty feeding worms.

Further evidence of a second mechanism, not involving nitrogen compounds, came from records of titrable acid during changes in volume of the medium (Table 7). Nothwithstanding the trivial changes in pattern of nitrogen output (p. 436, Table 5) there were striking changes in acid production, which therefore must have been buffered by non-nitrogenous alkali. *Lumbricus* also showed this effect to a smaller degree (Table 7, Table 4), and therefore probably also makes some use of this second mechanism.

The most probable non-nitrogenous alkali is $CaCO_3$ from the calciferous glands, which are particularly well developed in *Eisenia* (Stephenson, 1930, p. 127), and which appear to be concerned mainly with buffering and excretion (Robertson, 1936). Preliminary observations have indicated that *Einsenia* does leave more of

Table 7. *Titratable acid* (*μl.* N/100) *produced per g. per day by* Lumbricus *and* Eisenia *during the transitions* (1) *to increased volume of medium, and* (2) *to decreased volume,* (*a*) *when feeding and* (*b*) *when fasting,* (*i*) *in the medium as initially separated, and* (*ii*) *after conversion of any urea to ammonia*

Transition and condition	No. of records	Days prior to transition			Days following transition									
		3	2	1	1	2	3	4	5	6	7	8	9	10
Lumbricus														
1 (*a*) (i)	12	198	200	198	234	211	182	204	239	223	191	184	190	196
(ii)	12	207	226	259	261	266	266	259	254	240	236	238	241	237
2 (*a*) (i)	10	183	190	205	196	191	200	196	177	158	154	154	167	184
(ii)	10	243	231	247	205	186	200	200	180	175	184	200	217	219
2 (*b*) (ii)	7	−187	−120	−164	−270	−224	−312	−360	−326	−383	−168	−316	−330	−423
Eisenia														
1 (*a*) (i)	18	1065	1028	951	1429	1449	1547	1735	1815	1791	1752	1793	1907	1962
(ii)	18	1647	1479	1344	1781	2055	2075	2073	2018	2282	2664	2675	2607	2597
1 (*b*) (i)	10	962	696	737	1159	1050	975	914	825	873	900	866	859	—
(ii)	10	1172	993	967	1307	1332	1256	1083	1160	1403	942	826	—	—
2 (*a*) (i)	23	1640	1861	2005	1283	1024	1107	1144	1244	1208	1148	1031	938	890
(ii)	23	2418	2488	2473	1661	1391	1471	1548	1468	1446	1537	1599	1548	1454
2 (*b*) (i)	10	858	903	871	660	750	839	967	1084	814	846	801	884	—
(ii)	10	1057	897	826	679	935	1140	1255	1217	1243	1397	1281	1288	1294

soluble calcium salts in the medium than *Lumbricus*, which deposits relatively more of the insoluble $CaCO_3$ concretions.

Eisenia produced less urea as well as more NH_3 per unit weight than *Lumbricus* (Table 1), but it is surprising, perhaps, that the former should excrete *any* urea—if NH_3 is so urgently needed. However, the urea- and NH_3-producing mechanisms are partly independent (pp. 428, 439) and there may be an irreducible minimum of excretory urea. The tissues of *Eisenia* in fact proved to have an arginase activity of the same order of magnitude as those of *Lumbricus*. Moreover, the titrable acidity of the medium from *Eisenia* increased when its urea was converted to NH_3 (Fig. 6), so that urea itself may be used to buffer acid in some way—perhaps by forming urea-salts. Feeding *Lumbricus* also showed this phenomenon to some degree (Fig. 5 and Table 6). The increase in acid was not due to acid in the urease powder or to loss of NH_3 from the medium.

It is not certain, however, that urea has any normal significance for buffering in *Eisenia* since the titratable acidity of the medium was more constant, from day to day, after hydrolysis of the urea (Fig. 6) than as initially withdrawn from the worm. This is in sharp contrast to the records for *Lumbricus* (Fig. 5). In the latter, therefore, variations in urea output are an essential part of the buffering system but in the former, perhaps, irrelevant disturbances.

Eisenia resembles *Aphrodite*, *Hirudo* and *Sipunculus* in its relatively high NH_3 output (Prosser, 1950, p. 192) and in *Hirudo*, at least, this increases when fasting (Delaunay, 1931), so that this may be the primitive pattern (J. Needham, 1930) in Annelids, persisting in spite of the rather specialized habitat of *Eisenia* (p. 425). The pattern of nitrogen output in fact does seem to be rather conservative (Dresel & Moyle, 1950; Underhay & Baldwin, 1955). These workers have shown that terrestrial life does not necessarily immediately induce a decrease in NH_3 output, nor the return to aquatic conditions a decrease in urea output. It is not altogether surprising, therefore, that moderate changes in volume of the medium had little effect on the pattern of output of earthworms (p. 436), though it would be useful to learn more about the real nature and volume of the medium normally available to these worms.

In animals as diverse as man (Hawk *et al.* 1947, p. 722) and *Paramecium* (Cunningham & Kirk, 1941) NH_3 output increases when fasting, as it does in *Eisenia*. In man (Hawk *et al.* 1957, p. 971), and also in *Helix*, urea output decreases at this time so that the *Lumbricus* pattern is more completely reversed in man than in *Eisenia*. Nevertheless, mammals, like *Lumbricus*, use NH_3 to buffer acidity in the body, and there are probably many factors underlying the varied patterns of excretory nitrogen in animals. Pilgrim (1954) has indicated how complex may be the determination of this pattern, on considerations of energy economy alone.

In a number of responses (p. 433), and perhaps most clearly in the output of acid (Fig. 6), there were oscillations, with a regular periodicity of 4–6 days, relative to any change in external conditions capable of acting as a pulse generator. This oscillatory type of response by intact animals is probably very common (Needham, 1957).

SUMMARY

1. Up to one-half of the nitrogen excreted by both *Eisenia* and *Lumbricus* is protein, probably mucus. A high percentage of the remainder is urea and ammonia.

2. Leaf as food spares body nitrogen and reduces the total output of nitrogen, in three species of earthworm examined. A nitrogen-free diet of filter paper has little nitrogen-sparing action in *Lumbricus*.

3. The pattern of excretion of nitrogen compounds by *Lumbricus*, and especially the ratio ammonia/urea, varies with food-intake and this is the major factor found to affect the pattern.

4. Food causes an increased acid production by *Eisenia* and *Lumbricus*. The latter neutralizes this by increasing its output of ammonia, and decreasing that of urea.

5. The decrease in urea output is much greater than the increase in ammonia output, indicating some degree of independence between the two mechanisms.

6. When *Lumbricus* and *Eisenia* are kept in water, changes in volume, or in frequency of renewal, of the medium have little effect on the rate of output of ammonia or of urea, though the worms are affected by the accumulation of excreta in the medium.

7. By contrast to the nitrogen compounds, acid is liberated in proportion to the volume of the medium.

8. *Eisenia* differs from *Lumbricus* in the following respects: (*a*) it has a much higher specific output of total nitrogen and of the ammonia fraction; (*b*) ammonia constitutes a higher percentage of the total; (*c*) ammonia output does not decrease but increases when fasting; (*d*) it excretes considerable amino nitrogen; (*e*) it excretes more uric acid; (*f*) it has a higher specific output of acid and uses a non-nitrogenous base, possibly $CaCO_3$ from the calciferous glands, to neutralize much of this; (*g*) it makes little use of urea in the control of pH, or as a form of nitrogen excreted; (*h*) filter paper causes a considerable reduction in nitrogen output.

9. *Allolobophora caliginosa* has the *Lumbricus* pattern of output, with values of specific output much lower than those of *Lumbricus* itself.

10. Isolated posterior pieces of *Lumbricus* and *Eisenia* have a pattern of nitrogen output similar to that of feeding worms, but those of *Allolobophora* have the pattern of fasting worms of the species.

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THE INFLUENCE OF PIGMENT MIGRATION ON VISION OF HIGHER CRUSTACEA

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(With Plate 6)

INTRODUCTION

Exner (1891) divided the compound eyes of insects and crustaceans into two classes according to the distance separating the crystalline cones from the rhabdomes. In the apposition eye the proximal ends of the crystalline cones were contiguous with the distal ends of the rhabdomes, whereas in the superposition eye the crystalline cones were far removed from the rhabdomes. Exner believed that in an apposition eye a distinct image was always formed because only rays parallel to the axes of the ommatidia were received by it. On the other hand, the clarity of the image in a superposition eye depended on the position of the distal retinal pigment.

In the eye of an animal adapted to darkness, where the distal pigment was withdrawn to form a collar round the cones, the image was formed by rays refracted by the cones acting as 'lens cylinders', as well as by rays passing straight through. This Exner believed would give rise to a well-illuminated but blurred image because the focus of the rays would not be perfect. In the eye of an animal adapted to light, the distal retinal pigment moved proximally away from the cones. Exner believed that in this condition the oblique rays would be excluded so that, as in the apposition type of eye, only those rays parallel to the ommatidial axis would fall on the retina. The superposition eye could therefore form a sharp apposition image or a less distinct but brighter superposition image, depending on the position of the distal retinal pigment.

Exner considered that the function of the proximal retinal pigment of arthropods was to enhance stimulation in very weak light and to prevent overstimulation of the sensitive retina in very bright illumination.

Hassenstein (1954) attempted to compare the visual acuity of light-adapted and dark-adapted eyes in two species of prawns, *Leander serratus* and *Lysmata seticaudata*. He found no change in visual acuity whether the eye was in the dark- or light-adapted state. He conceded, however, that his experiments were not conclusive.

It seemed desirable, therefore, to test Exner's hypothesis on a number of Crustacea. The experiments described below were made on three species of prawns *Leander serratus* (Pennant), *L. squilla* (Linné) and *Pandalus montagui* Leach and on a mysid *Praunus flexuosus* (Müller). Species of *Leander* and *Praunus* were collected living amongst weeds in rock pools, while *Pandalus* was trawled from depths ranging from 6 to 40 fathoms. *Eupagurus bernhardus* (Linné), a hermit crab found on the shore and below low-water mark, was used to typify the behaviour of a crustacean with apposition eyes (Bröcker, 1935).

MORPHOLOGY AND OPTICAL PROPERTIES OF THE EYES OF *LEANDER SERRATUS, L. SQUILLA, PANDALUS MONTAGUI, PRAUNUS FLEXUOSUS* AND *EUPAGURUS BERNHARDUS*

The eyes of the first three species are essentially of the same pattern as that of *Palaemonetes vulgaris* Say, which has been fully described by Parker (1891). They are typical superposition eyes in which the crystalline cones lie at a considerable distance from the rhabdomes. The crystalline tract is a slender strand, the broader distal end of which is closely opposed to the lower surface of the cones. In this region formalin-fixed frozen sections reveal that the uppermost part of the tract is packed with clear globules, apparently of oil, which are lost during ordinary histological treatment. There is also a little red pigment in this region. The lower end of the crystalline tract is closely applied to the rhabdomes, though Parker (1891) states that it is not continuous with them.

If balsam-mounted unstained sections through the ommatidial axes are examined by the interference microscope, the crystalline tracts and the rhabdomes stand out as strongly refracting elements. They seem to be optically continuous with each other (Pl. 6). Sections of eyes of *Leander serratus*, cut transversely to the ommatidial axes, show that the proximal end of the crystalline tract becomes hollow and expands into four processes which lie just above the rhabdome.

Employing the interference microscope on $8\,\mu$ thick unstained sections we have observed that the crystalline cones have a slightly higher refractive index ($\Delta n = +0{\cdot}003$ to $0{\cdot}006$) than the surrounding balsam and are separated from each other by material of lower refractive index. The distal extremity of the crystalline tract referred to above has a refractive index which is only a little different from that of the surrounding balsam, but the rest of the crystalline tract and the rhabdome have relatively higher refractive indices ($\Delta n = +0{\cdot}015$ to $0{\cdot}030$). They are surrounded by a matrix scarcely differing in refractive index from the mounting medium. The cornea is also highly refracting. Thus, apart from a small region at the base of the cone, the crystalline cones and tracts form continuous strands of higher refractive index surrounded by media of lower refractive index. Balsam-mounted sections, cut transversely to the longitudinal axes of the ommatidia, fail to show any evidence that the crystalline cones refract more strongly at their central axes than at their periphery. Exner's hypothesis that they function as lens cylinders demands that the refractive index of the cone in the living eye should diminish from the centre outwards. These results therefore throw a little doubt on Exner's assumptions regarding the function of the cones.

Observations on fixed eyes cannot afford conclusive evidence of their condition in life. They may, however, give a good indication if the fixation is rapid and satisfactory, that is if the material responsible for refraction in the living eye is not disturbed and if the mounting medium replaces the body fluids originally present in the interstices and tissues. Since the optical path difference is approximately determined by the total mass through which the light passes (Barrer, 1956) the refractive indices of the various tissues, if well fixed, should have a similar relationship to one another, though not the same value, as they had in life.

Unfortunately, the material comprising the crystalline elements of the eye is gelatinous and unstable, so that it has not yet been found possible to study the optical properties of untreated ommatidia.

The eyes of the Decapoda Natantia and the mysid *Praunus flexuosus* contain three groups of pigment cells. Two of these groups contain a black pigment, probably melanin; these are the distal and proximal retinulae. These cells are grouped regularly about each ommatidium. The two distal pigment cells extend from the crystalline cones down to the basement membrane, but the pigment is most concentrated in a narrow band which varies in position. The eight proximal retinulae are arranged radially around the rhabdome and penetrate the basement membrane of the eye. One of them differs from the rest; its nucleus lies closer to the basement membrane than the nuclei of the other seven, and is placed excentrically in the Decapoda. None of these nuclei are covered by pigment since the proximal pigment always lies deeper down in the cells. Only the proximal pigment cells are innervated and these alone are believed to be the light-sensitive cells. Parker's use of the term distal retinulae is thus misleading. The third group of pigment cells, the accessory or reflecting pigment cells, are not arranged in relation to the ommatidial elements and are fewer in number than the ommatidia according to Knowles (1950). They contain a white amorphous reflecting pigment and their processes extend from below the basement membrane to the level of the bottom of the rhabdome.

The eye of the mysid *Praunus flexuosus* has been described by Mayrat (1956). It differs from that of the Decapoda Natantia in several respects. The crystalline cone is formed from two, not four, elements (Grenacher, 1879). The cones have a short crystalline tract and the nuclei of the proximal retinulae lie close to the cones. There are eight proximal retinulae and one differs from the rest, as in Decapoda. The nucleus of this eighth retinula, however, lies centrally in a clear space formed at the junction of the short crystalline tract and a prolongation of the rhabdome termed by Mayrat the epirhabdome. Mayrat calls the eighth retinula the 'accessory cell'; it should not, however, be confused with the reflecting pigment cells forming the tapetum which Parker calls the 'accessory pigment cells'. These cells are present in *Praunus* and their pigment forms a tapetum which migrates reciprocally with the dark pigment of the proximal retinulae, just as in Decapoda Natantia.

The anomuran *Eupagurus bernhardus* has no white reflecting pigment layer (Bröcker, 1935), though it possesses a golden pigment which gives a strong reflexion to rays falling obliquely to the axes of the ommatidia. This pigment is usually lost during histological treatment. It does not seem to occupy the same position nor to migrate as does the tapetal pigment of Decapoda Natantia. The eyes of hermit crabs, whether kept in light or in darkness, thus have a similar appearance.

BEHAVIOUR AND CONTROL OF THE RETINAL PIGMENTS

In a dark-adapted eye the distal pigment lies on either side of the crystalline cones, the proximal pigment lying entirely beneath the basement membrane while the reflecting pigment lies above it. During adaptation to light the distal pigment migrates proximally towards the rhabdomes, the proximal pigment migrates distally to envelop the rhabdomes, while most of the reflecting pigment migrates below the basement membrane. A small amount of reflecting pigment is left as a thin band just outside the basement membrane but entirely covered by dark proximal pigment.

An old and still unresolved problem is the method of control of the retinal pigments.

Welsh (1930*a*) observed that when dark-adapted *Palaemonetes* were given a short flash of light and then returned to darkness the distal pigment continued to move towards the light-adapted position. Kleinholz (1934) suspected therefore that the distal pigment might be under a hormonal control and devised experiments to test whether this was true. He injected the eye-stalk extracts of light-adapted *Palaemonetes* into dark-adapted animals in the dark and found that the distal pigment migrated proximally. Kleinholz (1936) found that the reflecting pigment also responded to the injections but not the proximal pigment. Welsh (1939) stated that by using greater concentrations of eye-stalk extract the proximal pigment also could be made to respond.

Kleinholz (1949) removed the sinus gland from specimens of *Cambarus* and found that the proximal pigment continued to respond to changes in illumination but not the distal pigment. This confirmed that the distal pigment was controlled by secretions of the sinus gland but threw doubt on the possibility of hormonal control of the proximal pigment. Knowles (1950) working on *Leander serratus* obtained exactly similar results.

The distal retinal pigment is known to display a diurnal rhythm under constant conditions of light or of darkness in a number of crustaceans. Evidence for rhythmic changes in the proximal pigment, however, is less well founded. It has been demonstrated in conditions of constant darkness in a proportion of species studied (Table 1), but has been claimed under conditions of constant illumination only in a single instance. In the majority of species the state of the proximal pigment depends only on the conditions of illumination at the time.

Though the evidence indicates strongly that a hormone controls the movements of the distal retinal pigment it is difficult to reconcile with this view certain observations by Parker (1897). He found that not only did both pigments in excised eyes respond to changes in illumination, but also that if part of the eye of an animal was covered, the pigments of the shaded ommatidia became dark-adapted though the rest of the eye was fully light-adapted. The only evidence for the hormonal control of the proximal pigment, on the other hand, is the injection experiment of Welsh (1939) and the few instances of rhythmic behaviour given in Table 1.

All the other results indicate that the proximal pigment is independent of sub-

Table 1. *Diurnal rhythm in the retinal pigments of crustaceans*

Crustacean	Distal pigment	Proximal pigment	Reflecting pigment
*Macrobrachium olfersii**	NL	—	—
*M. acanthurus**	NL	—	—
Cambarus virilis†	—	DD	—
Latreutes fucorum‡	—	NL	NL
Leander tenuicornis‡	—	—	NL
L. affinis‡	DD, NL	—	DD, NL
Penaeopsis goodei‡	F	DD	F
Anchistioides antiguensis§	DD, NL	—	DD, NL
Portunus anceps‖	DD	DD	Ab
P. depressifrons‖	DD	DD	Ab
Parthenope serrata‖	DD	DD	Ab
Calappa flammea‖	DD	DD	Ab
Trachypenaeopsis mobilis‖	NL	—	Ab or F
Eusicyonia n.sp.‖	—	DD	DD
Leander serratus¶	—	DD	DD

NL = dark-adapted at night in light;
DD = light-adapted during day in darkness;
— = no diurnal rhythm;
F = pigment shows no movement;
Ab = pigment absent.

* Welsh (1930*b*). † Bennitt (1932). ‡ Welsh (1935). § Welsh (1936). ‖ Kleinholz (1937). ¶ Knowles (1950).

stances circulating in the blood stream. Its behaviour during changes in illumination is certainly different from that of the distal pigment and appears to be a direct response to light.

Knowles (1950) studied the migration of the retinal pigments of *L. serratus* and *L. squilla*. He considered only the migrations brought about by changes from light to darkness and vice versa. Neither the diurnal movements of the pigments in constant light nor the rates of adaptation were dealt with. Moreover, no information is available on the behaviour of the retinal pigments of *Pandalus montagui* and *Praunus flexuosus*. For the purposes of this investigation it was therefore necessary first to make a thorough study of the behaviour of the retinal pigments of all these species.

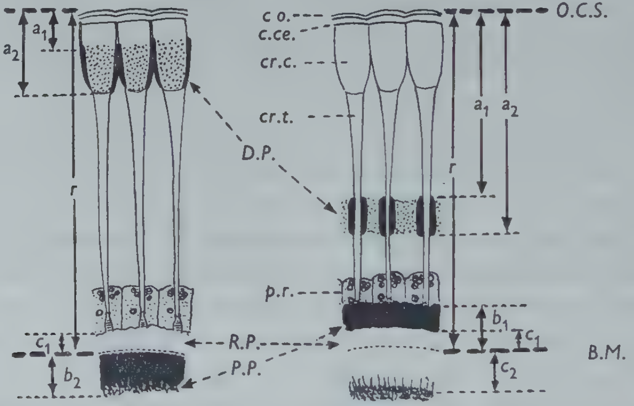
THE RESPONSES OF THE RETINAL PIGMENTS OF *LEANDER SETTATUS*, *L. SQUILLA*, *PANDALUS MONTAGUI* AND *PRAUNUS FLEXUOSUS* IN RELATION TO VARIOUS CONDITIONS OF ILLUMINATION

The changes in the retinal pigments were studied with the aid of sections of the eyes of animals treated in various ways and rapidly killed in very hot water. Sections were prepared using the technique recommended by Knowles (1950).

In order to define the position of the pigments independently of the size of the eye or of any shrinkage during fixation, an index for each pigment was introduced. The index in each case was derived from the distance separating a defined level of the pigment layer from a fixed level in the eye and was expressed as a fraction of the distance from the basement membrane to the outer surface of the crystalline cones.

The numerator of the distal retinal pigment index is the distance measured from the middle of the band of pigment to the outer surface of the cones. This index was always positive and increased in value as the eye became light-adapted.

The basement membrane affords the best reference surface for the proximal and reflecting pigments. These pigments may be found both proximally and distally to the basement membrane according to the state of the eye but unlike the distal pigment they do not migrate as a regular band. During light-adaptation the outer boundary of the proximal pigment moves in a regular and measurable way beyond the basement membrane but the boundary of this pigment below the basement membrane is ill-defined and the pigment here gradually fades. It is therefore desirable to differentiate between the behaviour of the pigment below and above the basement membrane by introducing two separate indexes, the one for outer surface of the pigment above and the other for the level of its inner boundary below the basement membrane. The indexes for the surfaces below the basement membrane are given a negative sign.



Text-fig. 1. Diagram of arrangement of retinal pigments in dark-adapted (left) and light-adapted state (right) to show how each pigment index is calculated. The outer corneal surface *O.C.S.* and basement membrane *B.M.* are the reference planes from which the distal (1) and proximal (2) limits of the pigment bands are measured. *D.P.*, distal pigment; limits a_1, a_2; *P.P.*, proximal pigment; limits b_1, b_2; *R.P.*, reflecting pigment; limits c_1, c_2. *co.*, cornea; *c.ce.*, corneagen cell; *cr.c.*, crystalline cone; *cr.t.*, crystalline tract; *p.r.*, proximal retinulae; *r*, distance separating reference planes O.C.S. and B.M.

The reflecting pigment is similarly treated, the levels above and below the membrane being recorded as separate indexes. The indexes may therefore be formulated as follows (see Text-fig. 1):

Distal pigment index $=(a_1+a_2)/2r$.
Proximal pigment index above membrane $=b_1/r$ (zero in dark-adapted eye).
Proximal pigment index below membrane $=-b_2/r$ (zero in light-adapted eye).
Reflecting pigment index above membrane $=c_1/r$.
Reflecting pigment index below membrane $=-c_2/r$ (zero in dark-adapted eye).

CONDITION OF THE PIGMENTS IN CONTINUOUS LIGHT AND CONTINUOUS DARKNESS

Freshly collected specimens of the four animals studied were divided into two groups. The first group was placed in a light-proof box in a dark room at a temperature ranging from 15 to 18° C. The water was kept well aerated with a diffuser in order to avoid any interference with the pigment responses due to lack of oxygen (Bennitt & Merrick, 1932). The second group was kept similarly aerated at the same temperature under illumination of 7·6 foot-candles (f.c.) in a glass dish.

The average pigment indexes of individuals removed at intervals are given in Tables 2–5. All four species behaved similarly.

It will be seen that in the dark the distal pigment index has its minimum value, i.e. it occupies its nearest position to the cones. The proximal pigment is entirely below the basement membrane and the reflecting pigment above it. In continuous light the proximal pigment lies entirely above and the reflecting pigment has partially migrated below.

It will be further noted, from an inspection of the sample estimates of the standard errors, that in no case is the proximal or reflecting pigment index significantly different between animals killed at night or during the day.

On the other hand, it will be seen that the values for the distal pigment index under continuous illumination are significantly higher in animals killed during the day than in those killed at midnight. The distal pigment therefore moves towards the dark-adapted position during the night even if kept well illuminated. The degree of dark-adaptation is greatest in *Praunus flexuosus* and least in *Pandalus montagui*.

RATES OF ADAPTATION OF THE RETINAL PIGMENTS ON EXPOSURE TO LIGHT

When exposed to light the distal pigment migrates inwards and the proximal pigment moves outwards covering the reflecting layer. The position of the outer boundary of the reflecting layer shows only a small change during light-adaptation, a thin layer always remaining between the basement membrane and the proximal pigment. It seemed reasonable to ignore changes which took place below the basement membrane as these could scarcely influence the response of the retinal receptors to light. We therefore concentrated attention on the migration of the proximal pigment distal to the basement membrane as it passed across the reflecting pigment layer and came to lie over it at the side of the rhabdomes. This movement is given by the proximal pigment index above the basement membrane. No attempt was made to follow the changes below the basement membrane as there was no clearly defined boundary to the pigment. The pigment gradually fades from this region as it becomes concentrated above the reflecting pigment.

Table 6 gives the values of the distal and proximal pigment indexes of three of the species studied after being exposed to illumination of 1·1 f.c. for various lengths

Table 2. Leander serratus

	No. of individuals	Distal pigment index	Proximal pigment index		Reflecting pigment index	
			Above	Below	Above	Below
Darkness						
Daytime	2	0·176 ± 0·023	0	0·244 ± 0·028	0·080 ± 0·012	0
Midnight	3	0·188 ± 0·012	0	0·240 ± 0·060	0·092 ± 0·005	0
Light						
Daytime	2	0·516 ± 0·025	0·136 ± 0·002	10	0·080 ± 0·001	0·272 ± 0·003
Midnight	7	0·287 ± 0·032	0·152 ± 0·014	10	0·092 ± 0·008	0·292 ± 0·009

Table 3. Leander squilla

	No. of individuals	Distal pigment index	Proximal pigment index		Reflecting pigment index	
			Above	Below	Above	Below
Darkness						
Daytime	2	0·200 ± 0·011	0	0·242 ± 0·028	0·130 ± 0·010	0
Midnight	4	0·192 ± 0·014	0	0·206 ± 0·030	0·108 ± 0·010	0
Light						
Daytime	4	0·481 ± 0·014	0·184 ± 0·010	0	0·086 ± 0·005	0·256 ± 0·016
Midnight	4	0·216 ± 0·032	0·146 ± 0·010	0	0·080 ± 0·008	0·258 ± 0·026

Table 4. Pandalus montagui

	No. of individuals	Distal pigment index	Proximal pigment index		Reflecting pigment index	
			Above	Below	Above	Below
Darkness						
Daytime	3	0·158 ± 0·003	0	0·118 ± 0·010	0·112 ± 0·004	0
Midnight	2	0·159 ± 0·009	0	0·110 ± 0·014	0·110 ± 0·014	0
Light						
Daytime	2	0·467 ± 0·014	0·119 ± 0·014	0	0·078 ± 0·002	0·222 ± 0·006
Midnight	2	0·317 ± 0·017	0·124 ± 0·004	0	0·070 ± 0·002	0·266 ± 0·046

Table 5. Praunus flexuosus

	No. of individuals	Distal pigment index	Proximal pigment index		Reflecting pigment index	
			Above	Below	Above	Below
Darkness						
Daytime	4	0·144 ± 0·012	0	0·260 ± 0·020	0·132 ± 0·008	0
Midnight	5	0·142 ± 0·010	0	0·286 ± 0·036	0·164 ± 0·024	0
Light						
Daytime	3	0·272 ± 0·040	0·172 ± 0·020	0	?	0·372 ± 0·044
Midnight	3	0·133 ± 0·014	0·136 ± 0·016	0	?	0·360 ± 0·042

Table 6. *Changes in the distal and proximal pigments after exposure to illumination*

Pandalus montagui

	Exposure time in minutes									
	1	2	3	4	5	6	10	15	30	∞
istal pigment index	0·141	0·138	0·127	0·130	0·115	0·166	0·130	0·145	0·303	0·467
roximal pigment index	0·020	0·042	0·046	0·090	0·020	0·110	0·108	0·126	0·150	0·121

Leander serratus

	Exposure time in minutes													
	2	4	6	8	10	15	20	25	30	40	50	60	90	∞
pigment x	0·166	0·155	0·185	0·185	0·177	0·225	0·225	0·297	0·418	0·439	0·442	0·468	0·486	0·515
nal pigment x	0·030	0·172	0·156	0·164	0·200	0·190	0·150	0·148	0·218	0·212	0·200	0·218	0·188	0·144

Praunus flexuosus

	Exposure time in minutes											
	2	3	4	5	6	8	10	15	20	25	30	∞
pigment x	0·192	0·186	0·163	0·182	0·204	0·207	0·234	0·250	0·272	0·280	0·280	0·272
nal pigment x	0·0	0·0	0·192	0·220	0·204	0·186	0·234	0·176	0·162	0·176	0·175	0·154

of time. To eliminate the effects of diurnal variations these experiments were conducted between 10 a.m. and 2.30 p.m.

There is considerable variation, as might be expected in unreplicated experiments, but the table shows that in all three species the proximal pigment migrates more rapidly into the light-adapted position than does the distal pigment. The last column of the table gives the index after prolonged exposure to illumination and is taken from Tables 2–5. The approximate time for the migration of the proximal pigment into the light-adapted state in *Praunus flexuosus* was about 4 min., for *Leander serratus* about 4 min., and for *Pandalus montagui* between 4 and 6 min. The corresponding times for the distal pigment to migrate into the fully light-adapted position were 20, 90 and more than 40 min. respectively. Moreover, the proximal pigment has reached its limiting position before any detectable change has occurred in the distal pigment. The proximal pigment below the basement membrane continued to diminish in quantity for some time after the outer boundary had reached its limiting position, so that some flow of pigment distally must have continued. However, this process does not lead to any noticeable increase in the density of the pigment around the rhabdomes which appears completely enveloped in dense black pigment after 4–6 min.

The independent behaviour of the retinal pigments allows experiments to be carried out to test Exner's views on the function of the distal pigment. Animals exposed for long periods to light during the day will have the distal and the proximal pigments in the light-adapted state. At night, however, the distal pigment will have migrated to some extent towards the dark-adapted condition, though the proximal pigment will remain light-adapted. Animals which have been fully dark-adapted and kept for a few minutes in the light will have the proximal pigment in the light-adapted state and the distal pigment will still be in the fully dark-adapted position. It is not possible to obtain the distal pigment in the light-adapted state while the proximal pigment remains dark-adapted.

VISUAL ACUITY OF INITIALLY DARK-ADAPTED ANIMALS

Specimens of *Pandalus montagui*, *Leander serratus* and *Praunus flexuosus* were first kept in complete darkness for at least 4 hr. A specimen was then removed from the light-proof box and placed at the centre of a cylindrical glass dish with a rotating striped cylinder outside. The visual acuity is expressed as the minimum angle between adjacent black and white stripes which causes an optomotor response. At the beginning of the experiment a cylinder with a small number of stripes was used. The light was then switched on giving an illumination of 1·1 f.c. at the bottom of the glass vessel. In this relatively weak light the dark-adapted animals did not show any distress or give an avoiding reaction. The time taken for the animal to respond to the rotatory pattern, either by swimming characteristically round the dish in the same direction as the rotation of the striped pattern or by nystactic eye-stalk movements, was recorded, and a cylinder with a larger number of stripes was quickly substituted. This resulted in the temporary cessation of the response. The time at which a response was again elicited was once more recorded and another substitution made. In this way the visual acuity was determined at intervals after initial exposure to the light. As a further check, in some experiments a cylinder with a larger number of stripes was used at the outset to confirm that the time at which the response was first obtained was independent of the order in which the patterns were offered. The results are shown in Table 7. It can be seen that there is some difference between individuals, but an improvement in visual acuity always occurs.

This takes place within 3 min. in *Pandalus montagui* and within 6–10 min. in *Leander serratus* and *Praunus flexuosus*. Text-figs. 2–4 illustrate how the visual acuity, the distal pigment index and the proximal pigment index vary with time after exposure to light for each of the three species studied. The figure demonstrates clearly that the visual acuity rises while the proximal pigment is migrating over the reflecting layer and surrounding the rhabdomes. The movement of the distal pigment which takes place afterwards has no influence on visual acuity.

Table 7. *Variation of visual acuity (in degrees) with time of exposure to illumination*

Pandalus montagui

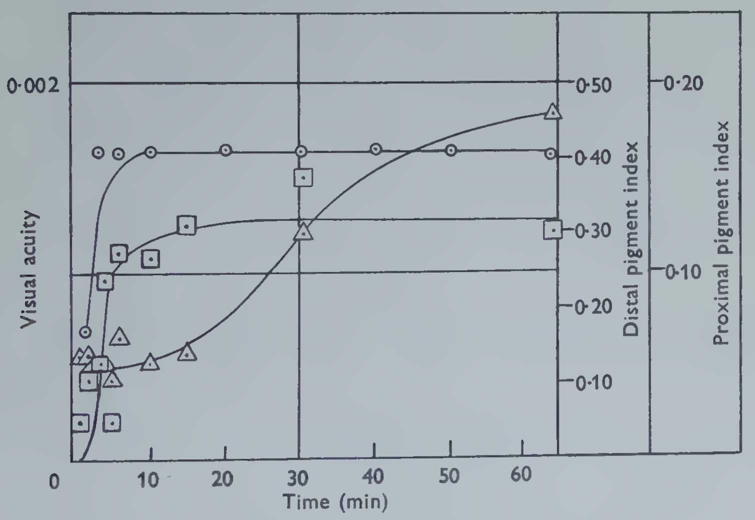
Pandalus no.	Exposure time in minutes							
	1	3	6	10	20	30	40	∞
1–3	23·1	10·9	10·9	10·9	10·9	10·9	10·9	10·9
4–6	19·5	9·5	9·5	9·5	9·5	9·5	9·5	9·5

Leander serratus

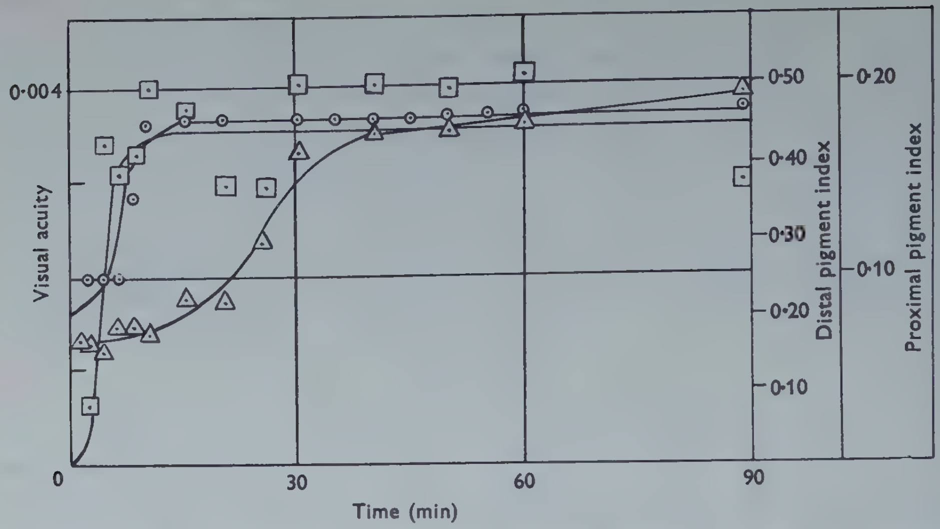
eander no.	Exposure time in minutes											
	2	4	6	8	10	20	25	30	40	50	60	∞
1	8·4	8·4	8·4	7·3	4·6	4·6	4·6	4·6	4·6	4·6	4·6	4·6
2–5	8·4	8·4	8·4	4·6	4·6	4·6	4·6	4·6	4·6	4·6	4·6	4·6

Praunus flexuosus

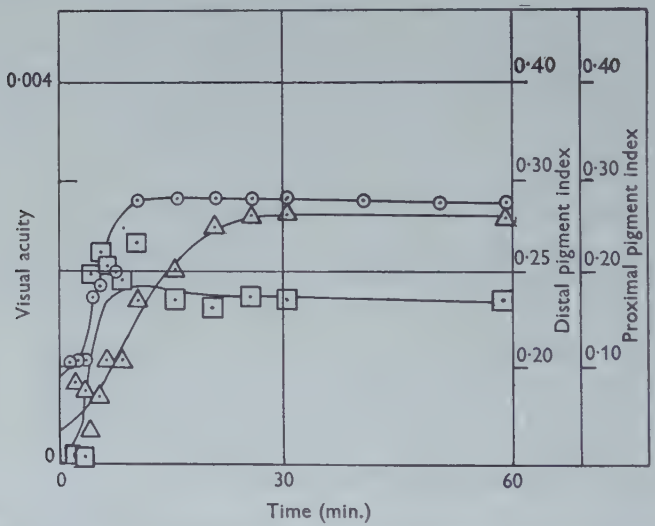
aunus no.	Exposure time in minutes												
	1	2	3	4	5	7	10	15	20	30	40	50	∞
1–3	16·5	16·5	16·5	10·9	9·5	8·4	6·2	6·2	6·2	6·2	6·2	6·2	6·2
4–6	16·2	16·2	16·5	8·4	8·4	8·4	6·2	6·2	6·2	6·2	6·2	6·2	6·2



Text-fig. 2. Effect of illumination on initially dark-adapted *Pandalus montagui*. The curves indicate the changes after a given time of illumination in the visual acuity, ⊙; the proximal pigment index, ⊡; and the distal pigment index, △. The visual acuity is given as the reciprocal of the visual angle in minutes as defined by Hecht & Wald (1934). Pigment index measured as described in text.



Text-fig. 3. Effect of illumination on visual acuity, proximal pigment index and distal pigment index of initially dark-adapted *Leander serratus*. Symbols and units as in Text-fig. 2.



Text-fig. 4. Effect of illumination on visual acuity, proximal pigment index and distal pigment index of initially dark-adapted *Praunus flexuosus*. Symbols and units as in Text-fig. 2.

VISUAL ACUITY DURING DIURNAL MOVEMENTS OF THE DISTAL PIGMENT

Freshly collected specimens of *Leander serratus* and *Praunus flexuosus* were kept under constant illumination of 7·6 f.c. for at least 2 hr. The visual acuity was measured, using the apparatus previously described, between 10 a.m. and 12 noon when the distal pigment was fully light-adapted, and between 12 midnight and 2.30 a.m. when the distal pigment was partially dark-adapted. Some of the animals used in these experiments were killed and the eyes sectioned to confirm the position of the pigments.

Table 8 gives the values of the minimum visual angle for individuals measured in the middle of the day and at midnight. There is clearly no difference in the visual angle caused by the shift in position of the distal pigment. Judging from the mean values of the distal pigment index, the migration of the pigment at night averages about two-thirds of the distance towards the fully dark-adapted state in *Leander serratus*, while in *Praunus flexuosus* the distal pigment is fully-dark adapted. Even in *Leander*, however, some individuals become fully dark-adapted at night; nevertheless, no change in visual acuity was ever observed.

Table 8. *Visual acuity (in degrees) during diurnal movements of the distal pigment*

Leander serratus			*Praunus flexuosus*		
Leander no.	Daytime	Midnight	*Praunus* no.	Daytime	Midnight
1	6·2	6·2	1	6·2	6·2
2	4·6	4·6	2	4·6	4·6
3	6·2	6·2	3	6·2	6·2
4	4·6	4·6	4	6·2	6·2
5	6·2	6·2	5	4·6	4·6
			6	6·2	6·2
			7	6·2	6·2
			8	6·2	6·2
			9	6·2	6·2
			10	6·2	6·2

VISION IN WEAK LIGHT

The following experiments were performed to test whether the dark-adapted eye was more sensitive than the light-adapted eye. Fresh specimens of *Leander serratus* and *Pandalus montagui* were used and also *Eupagurus bernhardus* since this species shows no mechanism for dark-adaptation (Bröcker, 1935). One group of animals was adapted to darkness by keeping them in a light-proof box for at least 3 hr. Another group was illuminated at an intensity of 1·1 f.c. for at least 3 hr. to ensure that they were fully light-adapted.

Each group was then tested with a broad striped pattern in which the alternate stripes were separated by an angle of 72 degrees. This was well within the limit of visual acuity. The test was carried out under a dark room safe-light (Kodak deep red iso-filter) which cut off all wavelengths below 6000 Å. The intensity of illumination was calculated to be $7{\cdot}2 \times 10^{-3}$ f.c. at the bottom of the vessel containing the animals. This light intensity was so low that the eyes of the observer required time to become sufficiently adapted before commencing the experiments. It was seen that only the dark-adapted individuals *Pandalus* and *Leander* were able to respond under these conditions. Evidently they are fairly sensitive to light of long wavelength. *Eupagurus bernhardus* gave no response at all, nor did light-adapted individuals of *Pandalus* and *Leander* (see Table 9).

The visual acuity of dark-adapted individuals of the two species which responded was then measured under the weak red light.

Striped cylinders of decreasing angular separation were rotated around the animals first clockwise and then anti-clockwise for 1 min. When no response was given after ten such trials the limit of visual acuity was considered to have been reached. Nystactic movements of the eye-stalks could not be seen, only body movements of the animals could be taken into account.

Table 9. *Visual acuity of previously dark- and light-adapted crustaceans measured in weak red light and in white light*

Visual acuity of	Red light > 6000 Å 0·0072 f.c.		White light 1·10 f.c.	
	Dark-adapted	Light-adapted	Dark-adapted	Light-adapted
Leander serratus	13·5°	No response	No immediate response	4·6°
Pandalus montagui	13·5°	No response	No immediate response	10·2°
Eupagurus bernhardus	No response	No response	4·6°	4·6°

The visual angle was found to be higher than that given by light-adapted animals in white light, showing that the greater sensitivity of dark-adapted animals in the weaker red light is associated with loss of resolution.

When this experiment was repeated under white light of 1·1 f.c., initially dark-adapted prawns did not respond at once; this we know is because when the pigments are dark-adapted the visual acuity is initially very low (see Text-fig. 1). After a short time, however, they became light-adapted and responded normally. *Eupagurus bernhardus*, on the other hand, responded immediately whether it had been previously kept in the dark or the light. Moreover, if the dark-adapted animals are exposed to an intense white light of about 500 f.c., these animals show a violent escape reaction, attempting to jump out of the dish. This reaction lasts 2–3 min. The light-adapted prawns and *E. bernhardus*, whether previously kept in the light or the dark, showed no discomfort when placed under strong white light.

THE FUNCTION OF THE RETINAL PIGMENTS

We shall first consider Exner's view that in the dark-adapted state a superposition image less distinct than the apposition image is allowed to reach the retinulae, but is prevented from doing so in the light-adapted eye by the distal pigment. This view can no longer be maintained because there is no change in visual acuity when the distal pigment migrates down from the cones. We must therefore examine how far this fact undermines the remainder of Exner's hypothesis. Two possibilities must first be distinguished according to whether the dioptric apparatus or the receptor elements limit the acuity of vision. If the dioptric apparatus is limiting, then it follows that the apposition image and the superposition image formed by it must have approximately equal resolutions. If, on the other hand, the retinal elements limit the visual acuity, as seems likely (de Bruin & Crisp, 1958), then we can only conclude that both images are sufficiently sharp to meet this requirement. A third possibility also emerges that the cones do not act as lens cylinders forming a superposition image as Exner supposed. The rays received by the retinulae would then be only those falling on the cone connected with them. The visual mechanism would therefore be the same whether the distal pigment were in the dark-adapted or in the light-adapted state. Such a possibility is consistent with the optical properties of the crystalline elements so far as can be judged from fixed preparations. Since their refractive index is greater than that of the surrounding tissues, they will act as 'wave guides' retaining by internal reflexion any ray whose direction is close to that of the ommatidial axis. These rays would pass down to the rhabdomes which would probably scatter the light around the adjacent retinulae.

Whichever of these alternatives is accepted the function of the distal retinal pigment is obscure. Since it does not affect the visual acuity, but obviously must restrict the light reaching the retinulae, it is natural to assume that its migration may function as a light-regulating device, perhaps to protect the retinulae from excessive illumination. The apertures through which light can enter the retinulae certainly become reduced as the distal pigment moves proximally, and as the pigment is concentrated over a much smaller area it becomes denser. There is as yet, however, no experimental evidence to support this view.

The proximal pigment cells and the reflecting pigment are best considered together since their functions are complementary. The dark pigment surrounding the rhabdome in the light-adapted state will clearly prevent light from being reflected back from the rhabdomes to the retinulae other than those immediately surrounding it. On the other hand, in the dark-adapted state the reflecting pigment lies immediately behind the rhabdomes, which are now no longer covered by black pigment. Stray reflexions can therefore pass in all directions. Bearing in mind the very small distances separating the bases of the ommatidia, these secondary reflexions will cause surrounding ommatidia to be stimulated in addition to those which are primarily illuminated. Thus the visual image will be less distinct. The black pigment therefore improves the visual image in a manner analogous to that of the anti-halation backing of a photographic plate. In the dark-adapted state the reflecting pigment increases the illumination falling on the retinulae though at the expense of definition. Our experiments in dim light show conclusively that dark-adapted animals are more sensitive to light, though this may be due not only to the reflexion of incident light by the accessory pigment cells but also to the adaptation of the photoreceptors themselves. However, this effect is not shown by *Eupagurus bernhardus* which lacks a tapetum. We have also shown that visual acuity is reduced when the reflecting pigment lies next to the retinulae, both in weak and in normal light intensities, though the latter soon brings the proximal pigment into the light-adapted position. It follows that the observed loss in visual acuity of dark-adapted animals is not related to the amount of illumination falling on the eye, as is sometimes the case (Hecht & Wolf, 1929), but is due to changes in the arrangement of pigments beneath the light-sensitive cells. This conclusion is borne out by the close parallelism between changes in the visual acuity and the position of the proximal pigment when dark-adapted animals are exposed to light.

SUMMARY

1. Exner believed that the movement of the distal pigment during light-adaptation improved the visual acuity of the superposition eye. This hypothesis was tested by measurement of the visual acuity of *Leander serratus*, *Pandalus montagui* and *Praunus flexuosus* under different conditions.

2. When a dark-adapted animal is placed in the light the proximal pigment migrates into the light-adapted position more rapidly than the distal pigment. The distal pigment, but not the proximal pigment, undergoes diurnal rhythm and tends to migrate into the dark-adapted position at night, even when the animal is illuminated. The visual acuity may therefore be tested when the distal pigment is still in the dark-adapted position and the proximal pigment in the light-adapted position.

3. No difference in visual acuity could be detected as a result of changes in the position of the distal pigment.

4. Visual acuity increases and light sensitivity decreases when the dark proximal pigment migrates over the reflecting (tapetal) layer.

5. *Eupagurus bernhardus*, which lacks the typical tapetum, shows no detectable change in visual acuity or sensitivity after being kept in the dark.

6. These experiments do not support Exner's view of the function of the distal pigment. They indicate that visual acuity is improved by the presence of the dark proximal pigment at the base of the proximal retinulae, probably because this pigment reduces stray reflexions from the back of the eye (halation).

7. It is suggested that since the crystalline cones and crystalline tracts form optically continuous strands with a higher refractive index than that of the surrounding medium they may act as wave guides. If so they would retain light entering the corneal surface from sources close to the axis of the ommatidium, and so concentrate it on the rhabdomes and adjacent retinulae.

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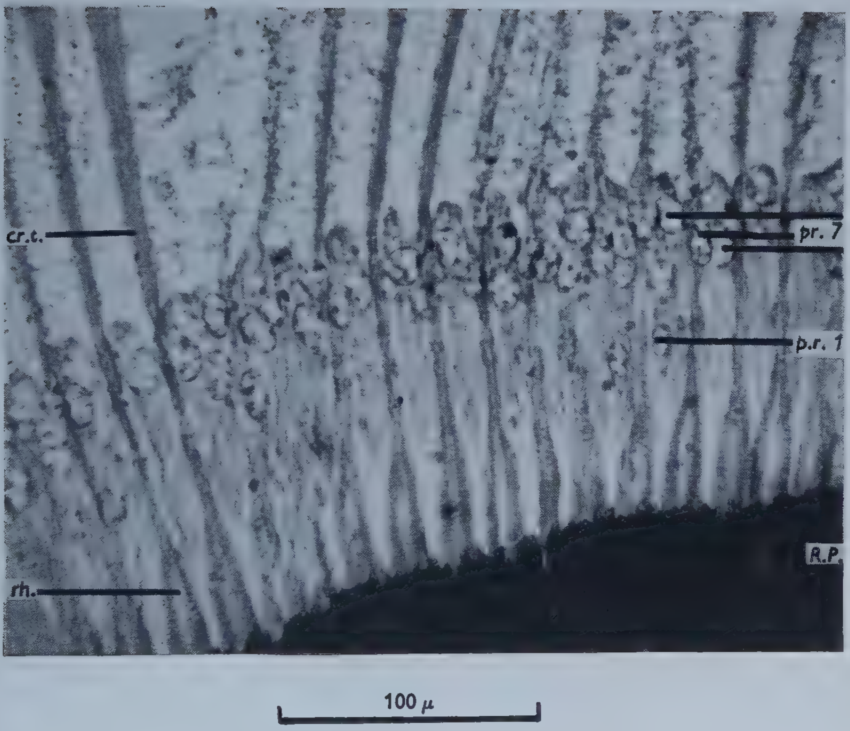

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EXPLANATION OF PLATE

Unstained sagittal section mounted in balsam of the eye of a dark-adapted *Leander serratus* viewed in the region of the crystalline tracts and rhabdomes under the interference microscope. The crystalline tracts and rhabdomes form a continous strand of highly refracting material, which appears darker than surrounding tissues. The rhabdomes also show bands approximately 2 μ apart, of lower refractive index alternating with bands of higher refractive index. *cr.t.*, crystalline tract; *pr.* 7, nuclei of proximal retinulae containing pigment (seven to each ommatidium); *p.r.* 1, nucleus of unique proximal retinula without pigment (one to each ommatidium); *rh.*, rhabdome; *R.P.* reflecting pigment.

EFFECT OF ANAESTHETICS ON WORKERS OF THE ANT *MYRMICA*

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INTRODUCTION

During recent experiments on ant populations it has been found convenient to anaesthetize worker ants. Anaesthetics have been used in apiculture, and it has been shown that the oviposition of queens (Mackensen, 1947) and the behaviour of workers of *Apis* (Ribbands, 1950, 1954; Simpson, 1954) may be significantly altered as a result. The experiments described below were carried out to determine the effects on worker ants of the anaesthetics commonly used on bees. The ant used was *Myrmica rubra* L., taken from colonies in the west of Scotland.

METHODS AND RESULTS

For the experiment described below ants were selected by occupation and were either nurses (*N*), domestics (*D*), or foragers (*F*). Nurses are approximately 4–6 months old, domestics 10–15 months old, and foragers more than 15 months old (Weir, 1958).

Workers were cultured in special containers (Brian, 1954), which represent optimal laboratory conditions. The experiment was carried out on five batches each of four culture tubes, each containing ten workers. Workers were kept in hibernation at 10° C. immediately prior to the experiment. Of the four cultures in each batch one consisted of segregated foragers, two of domestics, and one of nurses.

Worker oviposition (Brian, 1953), and worker survival, were the criteria used in the assessment of the effect of the anaesthetics. Eggs were removed each week and discarded. Observations were made on the workers at the same time.

Four of the five batches of cultures were treated, each with one of four anaesthetics, the fifth being used as a control. Anaesthetics used were carbon dioxide, nitrogen, nitrous oxide, and compressed air saturated with ether. Control cultures were subjected to a stream of compressed air. In the case of carbon dioxide, nitrogen, and nitrous oxide, each culture was subjected to the anaesthetic for 5 min. each day, timed from the moment that all workers in the culture had been immobilized. In the case of ether, cultures were subjected to the anaesthetics for only 1 min. after the immobilization of the workers. This was sufficient to keep workers narcotized or partially narcotized for periods up to 20 min. after removal of the gas source. After observation cultures were then subjected to a stream of compressed air for 2–3 min. to remove any residual anaesthetic. There was a 5-day break in treatment during the fifth week of the experiment.

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The weekly egg-output of each culture during the 7 weeks of the experiment is shown in Table 1. The results for the etherized cultures are omitted since no eggs were laid and all workers died within 7 days. Statistical analysis by the partitioning of variance shows that there are significant differences between the egg-outputs of the different kinds of workers. The egg-output of *N*-type workers is high, that of *D*-type workers moderate, and that of *F*-type workers low. Thus egg-output declines with age (Table 1). This change of egg-laying with age provides a basis for the detection of 'artificial ageing' (Janisch, 1924) of workers of *Myrmica*. With respect to the criteria used by Simpson (1954) for the detection of the effects of anaesthetics on *Apis* (glandular retrogression, worker disorientation on removal of the hive, and the inhibition of pollen collection), glandular retrogression was not investigated and the other two criteria are here not applicable.

Table 2 shows that except in the case of nitrous oxide, the total egg-output of cultures in all treated groups is significantly lower than that of the controls. 'Artificial ageing' as described by Janisch (1924) might then be assumed to have occurred in *Myrmica*. If this were so, then, as postulated by Ribbands (1950) in *Apis*, the expectation of life of the individual worker should be reduced. This, however, is no valid proof of ageing. Worker mortality is higher in all treated groups of cultures than in the controls (Table 3). Some of the differences in egg-output may be attributed to worker mortality, but no consistent relationship is demonstrable in this experiment.

Table 1. *Worker egg-output*

	Anaesthetic used															
	Carbon dioxide				Nitrogen				Nitrous oxide				Control compressed air			
Worker type	*F*	*D*	*D*	*N*	*F*	*D*	*D*	*N*	*F*	*D*	*D*	*N*	*F*	*D*	*D*	*N*
Week 1	.	.	.	.	.	.	.	.	.	3	4	6	.	.	.	.
2	.	.	.	.	.	.	.	.	4	4	8	10	.	.	.	.
3	.	.	.	.	.	.	3	3	3	12	13	17	.	6	9	12
4	.	.	.	.	5	24	.*	29	2	14	12	27	25	32	33	33
5	.	17	13	.	7	23	.	20	.	21	17	34	13	27	23	30
6	.	9	16	.	6	13	.	21	5	9	14	30	17	16	9	21
7	.	.	.	.	2	.†	.	5	.	7	15	12	14	.	.	6
Total egg production	.	26	29	.	20	60	3	78	14	70	83	136	69	81	74	102
Worker survival	8	6	4	3	3	0	.	5	7	5	9	6	9	7	7	9

* Culture accidentally destroyed.
† No workers alive after this week.

Further experiments on the side effects of ether and chloroform as anaesthetics were undertaken. The time of exposure to the anaesthetic was reduced to 30 sec.

per day. Worker survival was still low and no workers laid eggs or survived for more than 7 days. Observations on the individuals made about 1 hr. after anaesthetization on the third and fourth days of the experiment showed that many were by that time abnormal.

Table 2. *Total egg-output during 7 weeks of experiment*

Anaesthetic	Ether	Carbon dioxide	Nitrogen	Nitrous oxide	Control
Total egg output	Nil	55	161	303	326

Table 3. *Worker mortality after 7 weeks*

Anaesthetic	Ether	Nitrogen	Carbon dioxide	Nitrous oxide	Control
Percentage worker mortality	100*	73†	47	33	20

* 100 % mortality within seven days.
† Allowance has been made for the accidental destruction of one culture in this group.

DISCUSSION

Simpson (1954) concludes that nitrous oxide, carbon dioxide, and ammonium nitrate fumes (as smoker fumes), may affect worker bees in the following ways:—(1) by lack of oxygen; (2) by the presence of excessive quantities of carbon dioxide; (3) by the presence of substances as impurities in the production of nitrous oxide from ammonium nitrate smoker fumes. The third conclusion might be revised in the light of recent work on the combined action on crustaceans of two or more physiological poisons; Barnes & Stanbury (1948) on *Nitocra* and Hunter (1949) on *Marinogammarus* showed that more-than-additive (i.e. synergic) effects occurred in poisoning by mixtures of copper and mercury salts (respiratory and protoplasmic poisons respectively). Simpson (1954) suggested that certain anaesthetics may cause anaesthesia by interference with respiratory enzymes in *Apis*.

In the present experiments, the effect of lack of oxygen is seen in the cultures treated with nitrogen, where high worker mortality is coupled with low egg-output. The difference between these results and those for nitrous oxide treatment shows that the results of both these treatments cannot be attributed solely to oxygen lack. It appears that nitrous oxide itself has an effect on the workers; it causes premature egg-accumulation. The effects of nitrous oxide treatment are unlikely to be due to impurities since the gas was supplied in cylinders and conformed to medical standards of purity.

The effect of excessive carbon dioxide can also be distinguished from those of oxygen shortage or nitrous oxide treatment. The most striking effect is the complete suppression of egg-output until treatment is stopped for 5 days during the fifth week. It is interesting to contrast this result with that of carbon dioxide on queen bees, where the effect of a small number of doses is to accelerate oviposition (Mackensen, 1947).

Ether and chloroform, in the doses used, kill all workers within 7 days; both appear to have lasting and irreversible effects on the muscular co-ordination and nervous system of the workers.

It may be supposed that the effects of ether, carbon, dioxide, and nitrous oxide are here combined to varying extents with the effects of oxygen shortage. Nevertheless, each of the three anaesthetics has a separate and distinguishable effect on the workers.

If, as seems likely, anoxia effects are present when nitrous oxide is used, then the effect of the nitrous oxide may be partially to cancel the effect of oxygen lack, since both worker oviposition and longevity are better in the nitrous oxide-treated group than in the nitrogen-treated group. Nitrous oxide may even have a specific stimulatory effect on oviposition.

Janisch (1924) introduced the concept of 'artificial ageing' of insects following treatment with carbon dioxide. This concept was further investigated by Ribbands (1950), who showed that although the original concept was untenable with reference to *Apis*, normal changes in the behaviour of the worker might be accelerated by anoxial anaesthesia, whereas chloroform did not alter behaviour or longevity.

The experiments on workers of *Myrmica* described above allow measurement of any 'artificial ageing' by either egg-output or longevity. Ageing may be said to have occurred in cultures treated with nitrogen, carbon dioxide, and ether since egg-output in all cultures is reduced. In cultures treated with nitrous oxide, egg-accumulation starts 14 days earlier than in control cultures, so these also may be supposed to have 'aged' prematurely. In all groups, worker longevity was reduced after treatment. Therefore the requirements of the ageing hypothesis have again been fulfilled.

Nevertheless, these effects cannot represent true 'ageing' as postulated by Ribbands, since there is no detectable differential mortality between workers of different initial ages (*F*, *D* and *N*). Therefore the ageing hypothesis is untenable with respect to workers of *Myrmica*, for the same reason that Ribbands (1950) found it untenable in *Apis*.

It remains to consider the possible application to *Myrmica* of the hypothesis (Ribbands, 1950) that acceleration of changes in normal behaviour, caused by anoxia, would produce the observed results. All anaesthetics used show some effects on the workers, but these effects can be differentiated from that of anoxia. In respect of differences of egg-output one cannot differentiate between physiological effects (e.g. worker oviposition) and ethological effects (e.g. egg-eating by workers); worker egg-output is the resultant of all such factors. In this experiment longevity is not confused with environmental hazard, since the experiment was undertaken in optimal laboratory conditions. Thus, while different behaviour may lead to different metabolic rates under these conditions, changes in metabolic rate are unlikely to be sufficiently great to cause the differences here observed. These differential mortalities can then be attributed to the direct effect of the anaesthetic on the physiology of the insect.

As a result of these observations it is suggested that such effects as apparent ageing are the result of the direct action of anaesthetics on the physiology of workers.

This physiological interference may be reflected in behavioural changes. All anaesthetics used show this effect to varying extents, and none are completely satisfactory for experimental use.

The quantitative effects of these anaesthetics have not been investigated, but in the experiments described here, workers of *Myrmica* were exposed to repeated doses of anaesthetics, in contrast to previous work on *Apis* (Fyg, 1950; Gontarski, 1950; Ribbands, 1950; Simpson, 1954), where single doses or a few doses were given. This may well account for some of the differences observed.

SUMMARY

1. Experiments have shown that ether, nitrous oxide, and carbon dioxide all have significant and different post-anaesthesial physiological effects on workers of *Myrmica*. They reduce longevity and all except nitrous oxide reduce oviposition.

2. Nitrous oxide causes abnormally early accumulation of an egg mass.

3. The effects of these three anaesthetics can be distinguished from one another, and from the effects of nitrogen anaesthesia (presumed anoxia). Their effects may occur together with, and in addition to, those attributable to anoxia.

4. The effects of the anaesthetics on *Myrmica* longevity appear to be due to the direct physiological action of the anaesthetic and do not operate indirectly via ethological change as supposed in previous work on *Apis*.

5. These results are discussed in relation to previous work including the concept of artificial ageing which is shown to be untenable in this instance.

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REPEATED PRESENTATION EXPERIMENTS AND THE FUNCTION OF THE VERTICAL LOBE IN *OCTOPUS*

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INTRODUCTION

In a previous publication (Wells & Wells, 1957) it was shown that when octopuses with the vertical lobes of their brains removed are trained to make tactile discriminations, they make a much higher proportion of errors than controls. The increase in errors made can be attributed to a reduction in the ability to retain memories between trials, since it is possible to compensate for removal of the vertical lobe to a very considerable extent by arranging trials at shorter intervals. The present account is of some experiments made to determine whether there are other effects of removing the vertical lobe in addition to an increased rate of fading of memories.

MATERIAL

Octopus vulgaris Lamarck of between 300 and 800 g. from the bay of Naples were used in these experiments. The animals were caught and kept in individual tanks as described by Boycott (1954) and fed upon crabs or pieces of fish, those showing signs of damage due to rough handling at capture or failing to feed regularly being discarded. Each octopus was provided with three large bricks, piled together to form a 'home' at one end of its tank as described by Boycott & Young (1955). Animals not blinded (see next section) generally sat at the entrance to their homes, looking down the tank. Animals were not used in the experiments until they had been in captivity for several days and had ceased to react to the presence of an observer by retiring into their homes or by displaying the 'dymantic response' (Boycott, 1954). Some of the animals were then subjected to operations in which parts of their brains were removed; these animals were given a further post-operational period, during which they were fed regularly, before experiments were made with them.

In the text individual animals are referred to by the number with which they were identified in our original protocols, e.g. A 133. Numbers with the prefix A denote experiments made in 1954, those with B, 1955. The suffixes B, NOL, NV and NSF denote 'blind' (optic nerves cut but no other lesion), 'no optic lobes', 'no vertical lobe' and 'no superior frontal lobe', respectively.

OPERATIONS

Animals were anaesthetized in 3% urethane in sea water and lesions to the central part of the brain or removal of the optic lobes carried out as described in Wells & Wells (1957). Removal of the optic lobes involves section of the optic nerves and blinds the animal; lesions to the vertical or superior frontal lobe do not. One animal (A 106 B) was blinded by section of the optic nerves as described in Wells & Wells (1956).

Animals with brain lesions were killed at the end of the experiments and the lesions checked from serial sections. The extent of the lesion to the vertical lobe in the case of each individual so operated (estimated as in Boycott & Young, 1955) is given in Tables 1 and 2 as a percentage of the lobe removed. Maps of the lesions in the case of four animals (B 15 NV, B 16 NV, B 19 NV and B 39 NSF) that were subsequently trained to make tactile discriminations are given in Wells & Wells (1957). Removal of the optic lobes was invariably complete.

EXPERIMENTAL

When an octopus touches a small movable object that it has not previously encountered, it almost always grasps it and passes it to the mouth. In these experiments octopuses were required to learn *not* to do this with an inedible object presented to them. A series of objects of sealing-wax or Perspex were used; these objects have been fully described elsewhere (Wells & Wells, 1956) and are specified in the text, although their actual form is unimportant since the form of the objects, within wide limits, does not affect the results obtained. Animals were given no pre-experimental experience of the objects.

Experiments were carried out as follows: at each trial one object was presented by dropping it into the tank near the octopus under test. An octopus typically reacts to any small object arriving in this manner by reaching out an arm, grasping and passing it under the web to the mouth. Blind animals do the same when a small object is presented by gently touching it against an arm. In either case there follows a period during which the movements that the octopus makes suggest that it is attempting to break open the object as it would the valves of a lamellibranch shell. (Objects made of soft materials, such as weighted cork or plasticine, show signs of having been bitten when they are rejected. Evidently octopuses do not at first recognize objects as being inedible without trying to eat them.) After a period that may vary from about a minute to as much as half an hour (but is generally of the order of 2 min.), an object taken is finally rejected, generally being pushed away to arm's length. If the same object is repeatedly presented to the animal at short intervals (2 min. after rejection), the time taken to examine and reject it falls progressively until, commonly within a matter of three to five trials, a point is reached at which the octopus ceases to pass the object towards the mouth at all, rejecting it after a cursory examination with the arm tip lasting perhaps 5 sec. It appears that octopuses learn to recognize objects by touch before learning to recognize them by sight since most animals will continue to extend their arms to touch objects presented for many trials after they have ceased to give these anything but the most cursory tactile examination (see also Goldsmith, 1917).

Fig. 1 shows details of the performance of one animal (A 133) under these conditions. This animal learned to recognize a smooth sealing-wax cylinder (S 1) 2·0 × 2·5 cm. long so that this object was neither passed to the mouth nor even pulled towards the animal when touched after the 12th trial. From trials 13 to 23 no attempt was made to take the object, although the animal always reached out and examined it when presented. At trial 24, however, a different object, R 1 (which was like S 1 but had deep longitudinal grooves 0·5 mm. wide cut into it at intervals of 1·5 mm.) was presented and taken at once. This object was passed under the web to the mouth 3 times in the next four trials, the octopus thereafter ceasing (with one exception at trial 33, when R 1 was taken but at once rejected) to take the object. At trial 45 a third object, R 2 (with grooves 2·5 mm. wide 3 mm. apart) was introduced and taken twice in the next three trials before that, too, ceased to be taken when presented.

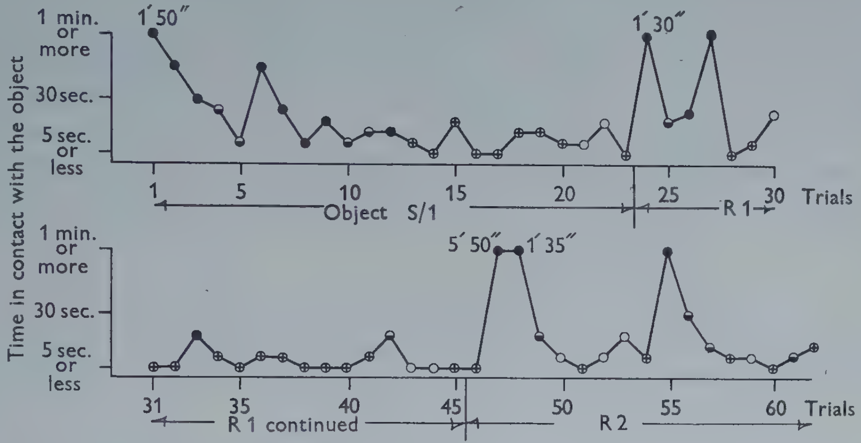

Fig. 1. Detail of the first part of the experiment with A 133 summarized in Table 1. Trials are at intervals of 2 min. 'Time in contact with the object' is the time elapsed between first contact when the animal reached out and touched the object and the final withdrawal of the arm from contact with the object. Reactions made are indicated as follows: ●, object passed to the mouth; ◒, object pulled towards the mouth before rejection or withdrawal; ⊕, withdrew (removed the arm after examining the object, leaving this where it was); ○, rejected (pushed the object away to arm's length). The second line of this plot follows upon the first after the usual 2 min. interval between trials.

Further examples of this sort of experiment are summarized in Table 1, which shows that a change of the object to one which the animal concerned had not already learned to reject in a previous set of tests, resulted in the new object being accepted in six out of seven cases; such experiments show that the animals are really learning to recognize the objects and not merely becoming generally less reactive or fatigued.

The results of experiments with two animals lacking the optic lobes are recorded in Tables 1 and 2; the performance of these animals was comparable to that of unoperated animals. This confirms the results of tactile discrimination experiments reported in Wells & Wells (1957), from which it was concluded that the optic lobes play no part in tactile learning, and in Table 2 animals lacking the optic lobes are included among the controls.

When repeated presentation experiments were made with animals having their vertical lobes damaged or destroyed, it was found that these could also learn to

Table 1. *Summary of 'repeated presentations' experiments showing retention following breaks in the sequence of trials at short intervals*

Animal	Object used	No. of trials	Trials at which the object was passed under the web to the mouth	Interval before the next series of tests if more than 2 min.
A 129*	S 1	49	1, 2, 11, 13	1 hr. 40 min.
	S 1	1	—	2 hr. 25 min.
	S 1	3	1	14 hr.
	S 1	4	—	—
	R 1	5	1	—
	S 1	1	—	4 hr.
	R 1	7	—	—
	R 2	6	1	—
	P 4	11	1, 2, 3, 4, 5, 6	—
A 133	S 1	23	1, 2, 3, 4, 6, 7, 8, 9, 12	—
	R 1	22	1, 3, 4, 10	—
	R 2	17	2, 3, 10	—
	S 1	5	—	—
	R 1	5	—	—
	R 2	5	—	3 hr.
	S 1	12	1, 4	—
	R 1	5	—	—
	R 2	5	—	24 hr.
	S 1	9	1	—
A 121 NOL	S 1	3	1	16 hr.
	S 1	11	—	—
	R 1	8	—	—
	S 1	1	—	25 hr.
	R 1	14	1	—
	R 2	10	1	—
	S 1	5	—	—
(9 days later)	S 1	20	1, 4	—
	R 1	5	—	3 hr. 30 min.
	S 1	10	—	2 hr. 30 min.
	S 1	5	—	64 hr.
	S 1	10	—	—
A 122 NOL	S 1	10	1, 2	2 hr.
	S 1	3	1	18 hr.
	S 1	8	2, 4, 8	—
A 128 NV	S 1	15	1, 2, 5, 6, 7, 8, 10, 11, 12	4 hr.
(100 % vertical	S 1	19	1, 2, 3, 4, 5, 6, 7, 8, 9	—
lobe removal)	R 1	3	1	16 hr.
	S 1	10	1, 6	24 hr.
	S 1	10	2, 4	74 hr.
	S 1	1	1	—
A 130 NV	S 1	30	1, 4, 6, 7, 9, 11, 13, 14, 15, 21	2 hr. 30 min.
(85 % vertical	S 1	24	1, 2, 3, 4, 5, 6, 7, 12, 13, 14	2 hr. 30 min.
lobe removal)	S 1	15	2, 6	64 hr.
	S 1	10	1	—

* Details of the performance of this animal are given in Wells & Wells (1956).

recognize and reject objects (Tables 1 and 2) and that, as in the case of controls, there was considerable individual variation in the number of trials required to do this. It can be seen from Table 2 that this number varies within a similar range for both controls and animals with vertical lobe lesions. It was also found that animals lacking parts of the vertical lobe tended to forget their training in the event of gaps in the sequence of trials at short intervals and were slower to relearn when training was resumed than controls which either did not forget or relearned very rapidly (Table 1). It is not possible to quantify these results because neither the number of trials nor the length of breaks in training were kept constant. A quantitative comparison between the times taken to reject the *first* object to be presented can, however, be made, and such measurements show that five animals having lesions to the vertical lobe* averaged 6 times as long as five controls to reject an inedible object on the first occasion that it was presented to them (average time in contact with the object 14 min. 19 sec. against 2 min. 23 sec. by controls). The movements that they made throughout this period, during most of which the object was out of sight beneath the web, indicated that they were trying to break open the object and not merely hoarding it.

Table 2. *Results of repeated presentations of the test object S 1*

(Only the first twenty trials (or less) of the first run of tests at 2 min. intervals is given. The times —to the nearest 5 sec.—given for each trial is the total time in contact with the object as in Fig. 1. Times in heavy type are occasions on which the animal passed the object under the web towards the mouth.)

	Controls					'No Vertical Lobe' animals					
al ...	A 106 B	A 121 NOL	A 122 NOL	A 129	A 133	A 128 NV	A 130 NV	B 15 NV	B 16 NV	B 19 NV	B 39 NSF
nt of vertical removed ...						100 %	85 %	60 %	60 %	90 %	100 %*
	m. s.	s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.
rial 1	**4 40**	**53**	**4 0**	**30**	**1 50**	**12 35**	**3 35**	**11 0**	**7 55**	**35 50**	**15 0**
2	**1 30**	10	**1 30**	**30**	**45**	**1 10**	10	15	**45**	**6 20**	15
3	**1 10**	5	35	5	**30**	30	5	10	10	10	**1 20**
4	**5**	—	20	5	**25**	5	**7 50**	10	40	20	15
5	**5**	—	10	10	10	**55**	5	10	20	**5 25**	**55**
6	**10**	—	25	10	**45**	**1 45**	**20 35**	5	10	5	5
7	**20**	—	30	5	**25**	**1 0**	**1 0**	10	5	15	45
8	**15**	—	10	5	10	**45**	25	5	—	10	15
9	**20**	—	5	5	**20**	10	**50**	—	—	5	10
10	5	—	5	5	10	**7 30**	10	—	—	5	10
11	10	—	—	**55**	15	**4 35**	**30**	—	—	—	**1 12**
12	5	—	—	5	**15**	**15**	20	—	—	—	45
13	5	—	—	**1 15**	10	10	**1 5**	—	—	—	20
14	5	—	—	5	5	5	**20**	—	—	—	5
15	5	—	—	5	20	5	**1 0**	—	—	—	5
16	5	—	—	5	5	—	25	—	—	—	5
17	5	—	—	10	5	—	10	—	—	—	10
18	5	—	—	5	15	—	5	—	—	—	5
19	10	—	—	5	15	—	5	—	—	—	—
20	5	—	—	10	10	—	5	—	—	—	—
f times the ct was taken	9	1	2	4	8	9	9	1	2	3	4
	4·8					4·7					

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* Plus one animal with a lesion to the superior frontal lobe, an operation which destroys most if not all of the afferent connexions to the vertical lobe (Boycott & Young, 1955) and can probably be regarded as functionally equivalent to complete removal of the vertical lobe.

DISCUSSION

The training technique used in the experiments reported in this account is subject to an important theoretical objection and to a number of practical difficulties. The use of unblinded animals in an experiment designed to test recognition of objects by touch is suspect because such animals could, in theory, learn to recognize the objects presented by sight or derive cues from watching the experimenter. In practice it can be shown that this defect in the experimental method is unimportant. It was not possible to train octopuses to distinguish by sight between two objects similar to those used in these tests (Wells & Wells, 1956), and it appears that under the conditions described in this account, octopuses learn to recognize an object by touch before learning to recognize it visually; they will continue to react by reaching out to grasp an object dropped near them for many trials after learning not to take it following subsequent tactile examination (Goldsmith, 1917, makes the same observation).

The most important practical difficulty is that of recovering the test object from the octopus's tank between trials. If the same object is to be presented after an interval during which the octopus has no possibility of experience of it, it must be removed from the aquarium when rejected. It is not always easy to do this without disturbing the animal, particularly if, as sometimes occurs, the object is rejected only to the edge of the web whence it is difficult to recover it without touching the animal. Octopuses that have become accustomed to life in aquaria tend to rush out and attack a hand, net or pincers moving in their tanks, and, disturbed by the struggle that inevitably follows, may retire home and fail to take objects presented during the next 10 or 20 min. An additional difficulty is that octopuses become very tame when kept in aquaria and rapidly learn to associate the presence of an observer with food, coming out of their homes and approaching down the tank when he appears. Animals that do this are useless for experiments that depend upon their staying in one place on the floor of their tanks. Another source of difficulty is the tendency of octopuses to hoard small objects, and some individuals will retain an inedible object presented to them for periods of hours or days before rejection. In such cases it is often possible to see the object held beneath the web, firmly grasped by the suckers, in the same position for hours on end.

The experiments reported in the present account are selected, being those in which none of these things occurred, the animals remaining in one place and continuing to reach out and examine objects throughout the tests. Nevertheless, they show a wide range of variation (e.g. A 129 learned not to take S 1 after two trials, A 133 only after twelve). This variation is probably at least in part a result of using untrained animals from a wild population in which individuals might well be expected to have had a wide range of different experiences in the sea before capture. It is likely that the number of times that the animal has been fed in the aquarium, what it has fed on, and how accustomed it is to the sight of the observer also affects the number of times it will accept an object presented before learning not to take it. Without making a large number of experiments it is impossible to evaluate the relative importance of these factors, and this was not done, the experimental method being abandoned in favour of a discrimination training technique that gives much more consistent results and is not subject to the same theoretical and practical objections (see Wells & Wells, 1956).

Nevertheless, certain general observations on the performance of animals in these tests may be made. These are:

(1) Under these conditions octopuses very rapidly cease to repeat actions that are not rewarded. In the present instance they learned not to take an inedible object repeatedly presented to them. Learning not to do this evidently involved recognition of a specific object, since when this was changed the octopus took the new object.

(2) Having learned to recognize and reject an object presented, octopuses remember for a period of hours or days, so that when the same object is again presented after such a break in training, it is either rejected at once or taken comparatively few times before the animal relearns to reject it (similar results are reported in Goldsmith, 1917).

(3) Animals lacking the vertical lobe, tested following similar breaks in training, showed comparatively poor retention of their initial training. This is in keeping with the results of comparable 'no reward' visual tests described by Boycott (1954) and with the results of tactile training experiments (Wells & Wells, 1957), both of which indicate that memory retention is comparatively poor in octopuses from which the vertical lobes have been removed.

(4) Provided that the sequence of trials at 2 min. intervals is maintained, animals lacking the vertical lobe take about as many trials as controls to learn not to take an object, but the time that they take to reject an object on the first occasion that it is presented is generally much longer.

This last result tells us something that discrimination training experiments cannot. It shows that removal of the vertical lobe has effects that cannot be attributed to a fading of memories between trials. The present data show that an animal lacking the vertical lobe must have unusually long experience of an inedible object before rejecting it on the first occasion that it is encountered and taken. This is not explicable on the basis that the vertical lobe removal has interfered with essential afferent or motor pathways since subsequent trials show that such animals can identify and reject the object just as rapidly as controls (Table 2). Nor is it likely that the behaviour of animals without vertical lobes in the first trial is due to an over-all increase in the 'positiveness' of response, as has been suggested to account for the results of vertical lobe removal in visual training experiments (Sutherland, 1957), since subsequent work has shown that such animals are *not* more likely than controls to react positively towards objects touched or seen (Wells & Wells, 1957; Young, 1957).

The nature of the deficiency shown can perhaps best be considered in terms of the changes that must take place in an animal's nervous system *during* the first trial.

It can be assumed, since the animal moves the object with its arms continuously throughout this period, that the pattern of the sensory input caused by the object remains more or less constant. The motor result of this input becomes modified during the period of the trial, so that finally rejection occurs instead of acceptance. Presumably this modification is brought about as a result of secondary sensory inputs, in the first instance from the mouth region when the animal tries to eat the object. In the case of animals lacking the vertical lobe this second input must continue for several times as long as normal before it can affect the motor consequences of the first input, implying that in the normal animal the vertical lobe serves to amplify the effects of such stimulation upon the organization of the animal's nervous system. It does this during the period in which sensory stimulation from the object is reaching the nervous system. This is of considerable importance in the interpretation of the results of discrimination experiments which indicate that the effect of each trial endures for longer if the vertical lobe is present (Boycott & Young, 1955; Wells & Wells, 1957). Such methods involve trials at each of which the animal is given a standard amount of experience (i.e. is exposed to the visual situation for 2 min., or allowed to react positively or negatively once to an object touched) and the results do not show whether vertical lobe removal decreases the effect of each trial or increases the rate of fade of this effect between trials, since in either case the deficiencies produced in training experiments would be most marked at low trial frequencies (see Wells & Wells, 1957). The present experiments strongly suggest that removal of the vertical lobe produces the deficiencies that it does because it renders each sensory experience less effective in the establishment of a memory at the time of its occurrence. If this is so, the vertical lobe in the intact animal is concerned more with the *establishment* of memories than with reducing the rate of fade of these memories between trials.

SUMMARY

1. When presented with a small object that it has not previously encountered, an octopus passes the object to its mouth and attempts to eat it. Inedible objects are rejected after a period of minutes, and when the same object is repeatedly presented at short intervals, the animal rapidly learns to examine and reject it without passing it to the mouth.

2. That the animal is learning to recognize a particular object and not merely becoming fatigued or generally less reactive can be demonstrated by changing the object; a new object is almost always taken, even if the animal has not taken the original for many trials.

3. After a break in training of the order of hours, the animal may accept the same object when it is presented again, but relearns not to take it in fewer trials than in the original training.

4. The performance of two animals tested after removal of the optic lobes was in all respects comparable to that of three controls (two unoperated and one with the optic nerves cut).

5. Six animals with the vertical lobe of the brain removed were also tested. The performance of these animals differed from that of five controls in two respects:

they took six times as long to examine and reject the objects in the first trial, and they showed relatively poor retention following breaks in training. The first of these differences is particularly important because it shows that removal of the vertical lobe has effects other than increasing the rate of fading of memories between trials. The significance of this in the interpretation of the function of the vertical lobe from discrimination experiments is discussed.

The experiments reported in this paper were carried out at Naples at a time when M. J. W. was a member of the staff of the Stazione Zoologica di Napoli and also holding an Eli Lilly Fellowship. The work was prepared for publication in Cambridge during the tenure by M. J. W. of a Fellowship at Trinity College. The authors would like to thank Prof. J. Z. Young, F.R.S., and Mr B. B. Boycott for helpful criticism during the work and for reading the manuscript.

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GLUCOSE ABSORPTION IN THE COCKROACH

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INTRODUCTION

The physiology of absorption from the gut in insects has been little studied. In particular there is a paucity of information on the absorption of nutrient substances from the gut. Much of what is known on this subject is qualitative in nature, and has usually been indirectly derived from specialized investigations on digestion or storage of nutrient materials. The majority of these studies have been adequately summarized in the review by Waterhouse & Day (1953).

In the present investigation the absorption of ^{14}C-labelled glucose from the gut of the adult cockroach (*Periplaneta americana*) has been studied, using a dye, Amaranth, as a marker. The dye is not absorbed from the lumen of the cockroach gut and thus the percentage absorption of glucose can be calculated from the glucose/dye ratio in the various parts of the gut. This technique has been recently used by Reynell & Spray (1956*a*) using phenol red as a marker to study intestinal absorption in the rat. Phenol red is, however, unsuitable for the study of poorly absorbed substances, since these authors were only able to get a 90 % recovery of the dye from the intestine. Amaranth, at least in the cockroach, is not limited in this way, for it was possible to recover this dye quantitatively.

METHODS

In order to obtain individuals with the gut empty, adult cockroaches were isolated in glass jars and deprived of food for 1 week. They were given access to water except for the 24 hr. immediately preceding the experiment. Each cockroach was then allowed to drink 0·10 ml. of an experimental solution which was offered to it on a small planchette. After an appropriate period the insect was killed by immersion in boiling water, a procedure which appeared to produce no violent movements of the gut. The whole gut was then excised, quickly washed in saline and after ligaturing cut into appropriate portions, each of which was dropped into a known volume of a solution buffered to pH 10·0. These portions were homogenized and centrifuged at 4000 r.p.m. until a perfectly clear supernatant was obtained for analysis.

The experimental solutions contained 0·008 M/l. of the dye Amaranth (Azo-Rubrin S) to which were added varying amounts of the test substances used. The concentration of this dye was determined in solution at pH 10.0 using a Unicam absorptiometer at an absorption maximum of 510 mμ.

The ^{14}C-labelled glucose was assayed using a thin-windowed Geiger-Muller tube (G.E.C. CV 2139). 1·0 ml. of the radioactive solution was pipetted into a small glass container consisting of a short length of 3·7 cm. diameter glass tubing closed at one end by a sheet of 0·00025 in. terylene sheet cemented on with 'Araldite'. The terylene sheet thus formed the floor of a cylindrical cell which was placed over the window of the G.M. tube. Because of its extreme thinness the stopping power of the terylene sheet was relatively low even to the low energy particles emitted by ^{14}C.

The insects used in this investigation were reared and maintained during the experimental period at a temperature of 28·0 ± 1·0° C.

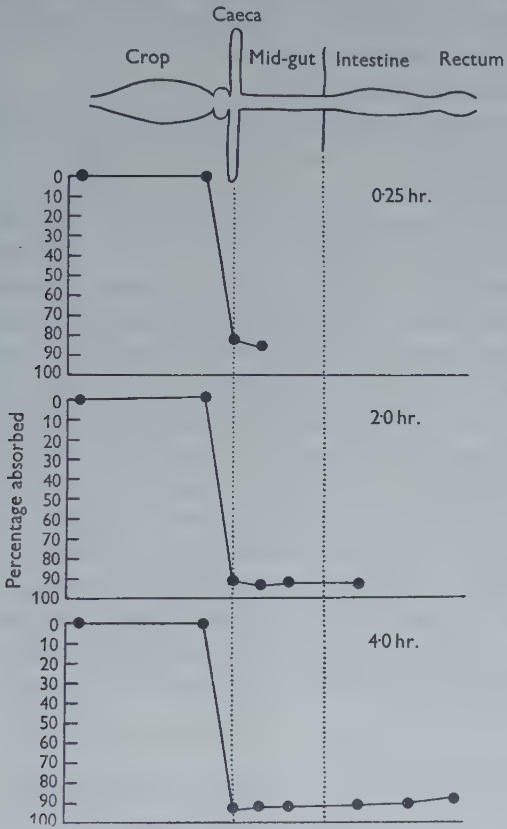

Fig. 1. The percentage absorption of glucose from various parts of the gut at three time intervals.

RESULTS

The dye Amaranth, which was used as a marker in these experiments, was selected from a number of substances which were tested. In a series of tests it was found that the recovery of Amaranth from the intestine, 6 hr. after feeding measured amounts of the dye, was 99·07 ± 1·80 %. It was concluded, therefore, that this dye was not absorbed from the lumen of the intestine, neither was it absorbed in significant amounts on to the gut wall. In the present study the dye has been used to measure transit in the gut and, by comparing the ratio of dye to glucose in various parts of the gut, to determine the net percentage glucose absorption.

In order to establish the site of glucose absorption in the cockroach gut starved adult insects were fed 0·10 ml. of a solution of 0·008 M/l. Amaranth and 0·05 M/l. ^{14}C-labelled glucose. The percentage absorption in the various parts of the gut has been calculated from the glucose/dye ratio. Fig. 1 shows the amounts of glucose absorbed as the solution reached different regions of the gut after 0·25, 2·0 and 4·0 hr. It is clear that most of the glucose had disappeared from the lumen of the gut by the time the solution had reached the mid-gut caeca. There was no uptake from the crop and there appeared to be no significant absorption as the remaining glucose passed along the rest of the gut.

As the glucose absorption appears to be largely confined to the mid-gut caeca it is clearly of importance to know something of the rate at which the glucose solution reaches these absorptive surfaces. Some light has been thrown on this process by studying the rate of crop emptying. In these experiments starved insects were fed 0·10 ml. amounts of various glucose solutions containing 0·008 M/l. Amaranth. These individuals were killed at intervals after feeding and from the amount of dye remaining in the crop the volume of the crop contents was determined. The concentration of the dye in the crop did not change during the experimental period, indicating that there was no appreciable dilution of the crop contents by liquid from the saliva or the mid-gut. Fig. 2*a* shows the volume of the crop contents at various times after feeding the insect 0·01 ml. of a 0·11 M/l. glucose solution. If these volumes are plotted on a logarithmic scale with respect to time (Fig. 2*b*) then a straight line is obtained. Crop emptying is thus an exponential function of time. In this experiment the half-time for crop emptying was 2·73 hr. To study the effect of glucose concentration on crop emptying cockroaches were fed 0·10 ml. amounts of solutions at varying glucose concentrations. In Fig. 3 a semilogarithmic plot illustrates the rate of crop emptying at glucose concentrations of 0·11, 0·55 and 2·77 M/l. The rate of crop emptying is thus slower at the higher concentrations of glucose. The half-time for crop emptying was calculated according to the relation $t_{0\cdot5}=\frac{t\times 0\cdot3010}{\log_{10} v/v_1}$, where v is the initial volume and v_1 that remaining in the crop after time t. It will be seen that $t_{0\cdot5}$ varies linearly with glucose concentration (Fig. 4).

The effect of osmotic pressure on crop emptying was studied by feeding cockroaches with solutions of different substances at similar osmotic pressures. The substances which were compared with glucose in this respect were sorbose, glycerol and sodium chloride. The results, which are summarized in Table 1, show that there was no significant difference in the half-time for crop emptying between any solutions of the substances used. Experiments were also performed to demonstrate the effect of one of these substances on glucose absorption. To do this one batch of starved individuals were fed 0·10 ml. of 0·05 M/l. glucose solution, while a second batch were given a similar solution containing in addition 0·20M/l. sodium chloride. After 1·0 hr. the whole gut was removed and the amount of glucose absorbed was calculated from the amount remaining in the gut. The results are summarized in Table 2.

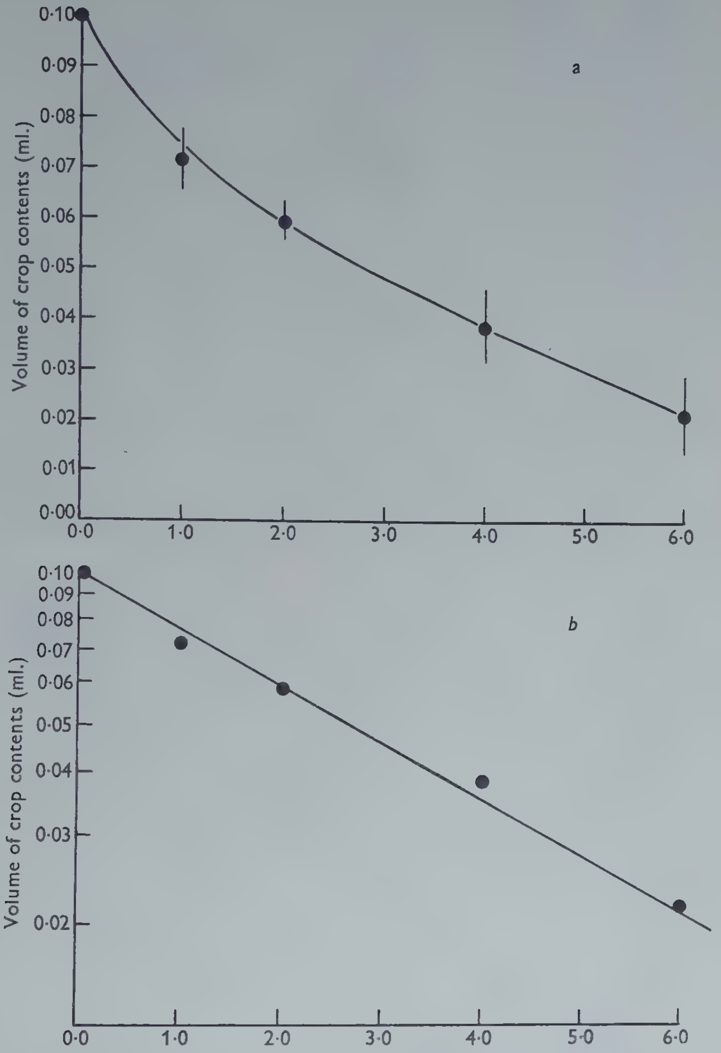

Fig. 2. (*a*) The volume of the crop contents plotted against time. Each point is the mean of five determinations, the vertical lines represent the extent of the standard deviation. (*b*) A semi-logarithmic plot of crop volume with respect to time.

The above experiments suggested that the rate of crop emptying might have a profound effect on the total glucose absorption by the insect. This effect was investigated by feeding starved insects 0·10 ml. volumes of the dye solution containing ^{14}C-labelled glucose. After suitable experimental periods the amounts of dye and

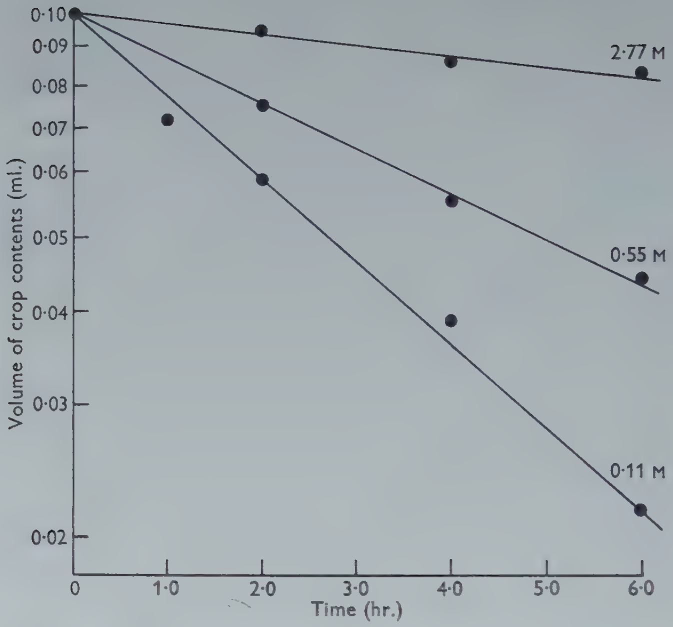

Fig. 3. The effect of glucose concentration on the rate of crop emptying.

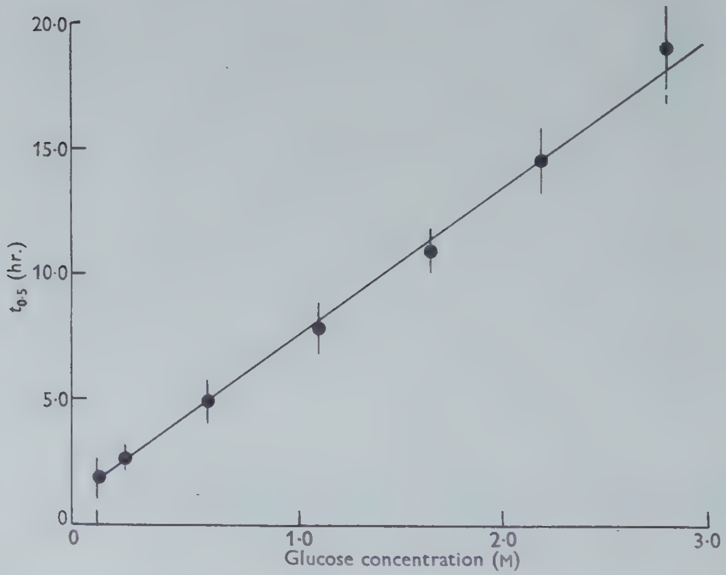

Fig. 4. The relation of the time for half emptying of the crop ($t_{0 \cdot 5}$) with glucose concentration. Each point is the mean of at least five determinations, the vertical lines represent the extent of the standard deviation.

Table 1. *The effect of different substances at similar osmotic pressures on the half-time of crop emptying*

Substance	Concentration (M/l.)	$t_{0\cdot5}$ ± s.d. (hr.)
Glucose	0·55	4·97 ± 0·83
Sorbose	0·55	5·01 ± 0·91
Glycerol	0·55	5·35 ± 0·95
Sodium chloride	0·277	5·08 ± 1·09

Table 2. *The effect of the presence of sodium chloride on glucose absorption*

Solution	Glucose absorbed (μg ± s.d.)
0·05 M/l. glucose	374·8 ± 21·8
0·05 M/l. glucose + 0·20 M/l. NaCl	149·1 ± 23·3

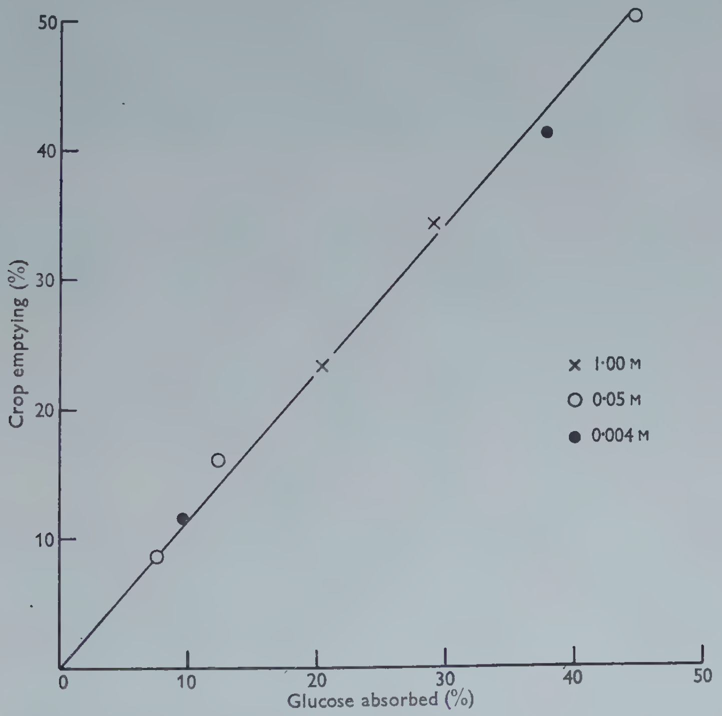

Fig. 5. The relation of crop emptying to glucose absorption at three concentrations of glucose.

glucose in the gut were measured. The degree of crop emptying was determined from the amount of dye present in the crop, and the total amount of glucose absorbed was calculated from the glucose recovered from the crop and the remainder of the gut. The results of these experiments, which are illustrated in Fig. 5, show the relation of the percentage crop emptying to the percentage glucose absorbed from solutions at three different concentrations. These results show that a linear relationship exists between glucose absorption and the rate at which the solution leaves the crop.

DISCUSSION

It is clear that in this insect glucose absorption is confined to the mid-gut caeca, for no appreciable uptake could be demonstrated in any other part of the gut. There has in the past been some controversy as to the part played by the crop in the absorption of sugars. This will be dealt with in a subsequent paragraph.

The rate of crop emptying was shown to be linearly related to the concentration of the ingested glucose solution, so that the amount of fluid leaving the crop decreased with increasing concentration. The total amount of glucose absorbed also showed a linear relation with crop emptying. Thus the limiting process in the absorption of glucose is not the transfer of the substance across the intestinal wall, but the rate at which the liquid is allowed to leave the crop. Such a system would operate to prevent the saturation of any glucose-absorbing mechanism in the gut. This state of affairs can be compared with that found in mammals, where it has recently been shown in the rat that the rate of gastric emptying decreased with increased glucose concentration (Reynell & Spray, 1956*b*). The percentage glucose absorption was also found to be related to gastric emptying (Reynell & Spray, 1956*a*).

The effect of concentration of the ingested solution on the crop emptying would seem to be an osmotic effect, for it was shown that the rate of emptying remained constant when different substances were tested at the same osmotic pressure. The cockroach differs in this respect from the rat where the rate of gastric emptying could not be directly related to osmotic pressure (Reynell & Spray, 1956*b*).

An effect resulting from this control of crop emptying in the cockroach is that a substance in solution can affect the absorption of a second substance, by reducing the volume of fluid available for absorption in the mid-gut. It was shown that the addition of 1·17% sodium chloride to a 0·05 M/l. glucose solution resulted in a 60·2% decrease in the amount of glucose absorbed.

It will now be appreciated why many of the early workers (e.g. Sanford, 1918) considered that the crop was responsible for sugar absorption. With their qualitative tests they could not detect appreciable amounts of sugar in the mid-gut, and concluded that it had not reached this part of the gut from the crop. Yet the sugar in the crop clearly decreased and therefore, they argued, it followed that it was being absorbed by this organ. The present investigation has shown that crop emptying is the limiting process in glucose absorption, so that the glucose in the mid-gut is always at a very low level, while there is a continuous decrease in the total amount held in the crop as it passes to the mid-gut. The possibility that this might be the explanation was appreciated by Cuènot (1896, p. 307). It has also been shown by Abbott (1926) that the isolated crop wall was relatively impermeable to glucose.

SUMMARY

1. The absorption of glucose from the gut of the cockroach has been studied by feeding starved insects with ^{14}C-labelled glucose together with a dye, Amaranth. This dye is not absorbed from the lumen of the intestine and the net percentage glucose absorption has been calculated from the glucose/dye ratio in the various parts of the intestine.

2. Glucose absorption is largely confined to the mid-gut caeca.

3. The rate of crop emptying, which is an exponential function of time, is related to glucose concentration, so that the amount of fluid leaving the crop decreases with increasing concentration. This effect is determined by the osmotic pressure of the ingested fluid.

4. The total glucose absorption shows a linear relation with crop emptying, suggesting that crop emptying is the limiting process in glucose absorption in the cockroach.

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A TEMPERATURE RECEPTOR IN THE TARSUS OF THE COCKROACH, *PERIPLANETA AMERICANA*

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INTRODUCTION

During the course of experiments on the reactions of the cockroach, *Periplaneta americana*, to temperature changes (Kerkut & Taylor, 1956) we noted that when the floor of the vessel containing the insect was cooled, the cockroach became more active. The activity later ceased and the animal rested on the floor of the container. After a little while the insect took up a characteristic position with the claws and distal ends of the the tarsi raised from the ground; the insect's weight resting on the proximal joints of the tarsi (Fig. 1).

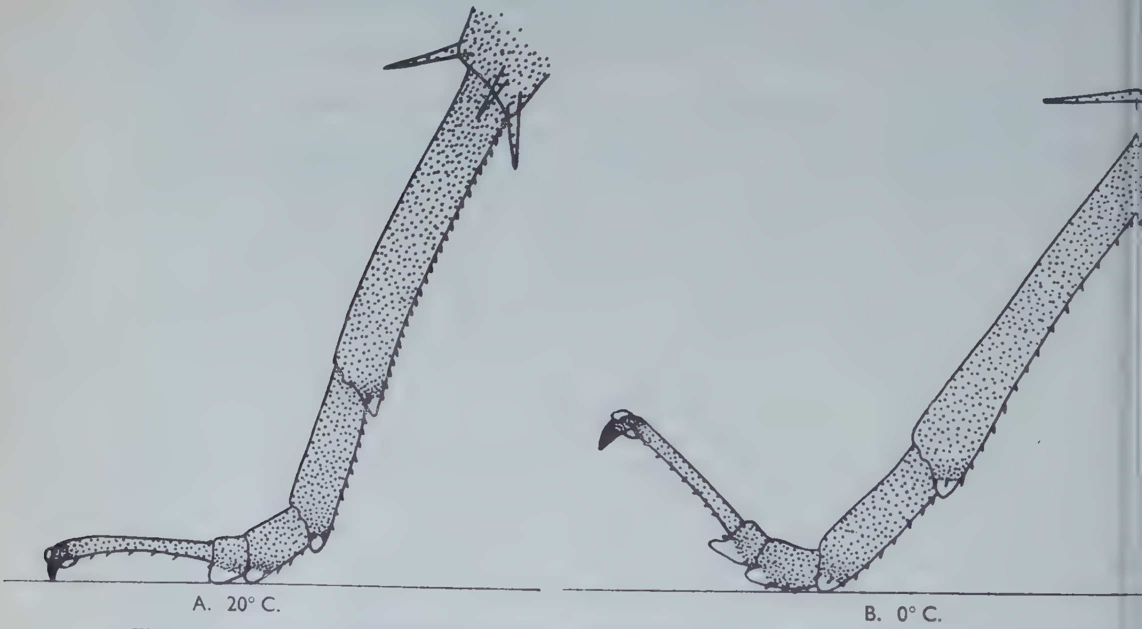

Fig. 1. Diagram of the position of the distal end of the cockroach's leg on surfaces at different temperatures. (A) On a surface at 20° C. (B) On a surface at 0° C.

This reaction was so consistent that it seemed possible that there might be a temperature receptor in the distal part of the tarsus and that the insect was removing the sense organ from the source of stimulation. It was therefore decided to see what effect cooling the tarsus had on the electrical activity of the nerves in the leg.

METHOD AND APPARATUS

The apparatus consisted of a Perspex box fitted with a stainless steel lid. Water or cooling mixture at a specific temperature was drawn through the box by means of a water pump. The temperature of the water was recorded by means of a thermometer and a thermistor placed immediately below the steel lid. The internal volume of the box was low due to the presence of an internal baffle; this facilitated a rapid change in the temperature. Changing the temperature did not lead to any detectable buckling or movement of the part of the steel roof in contact with the insect's tarsus.

The cockroach leg was removed from the animal and placed so that the distal end of the tarsus rested on the metal surface of the apparatus. A pair of platinum recording electrodes were placed into the tibia as near the leg nerves as possible, and the electrical activity noted. The potentials were amplified in a Leak TL 12 amplifier and recorded on a Ferrograph Tape Recorder. The written records were made on an Ediswan Pen Recorder.

RESULTS

In many preparations we found that after a few minutes there was no background activity. In others there was some background activity which increased if the apparatus was tapped. This was clearly due to stimulation of mechanoreceptors and we then adjusted the position of the leg on the apparatus till there was no background activity. Fig. 2A shows the effect of lowering the temperature. There was no activity before the temperature change, but when the temperature was lowered from 22° to 2° C. there was a sudden burst of activity which lasted approximately 12 sec. Fig. 2B shows the effect of raising the temperature of the same preparation. The temperature was raised from 2° to 22° C. and there was a short burst of

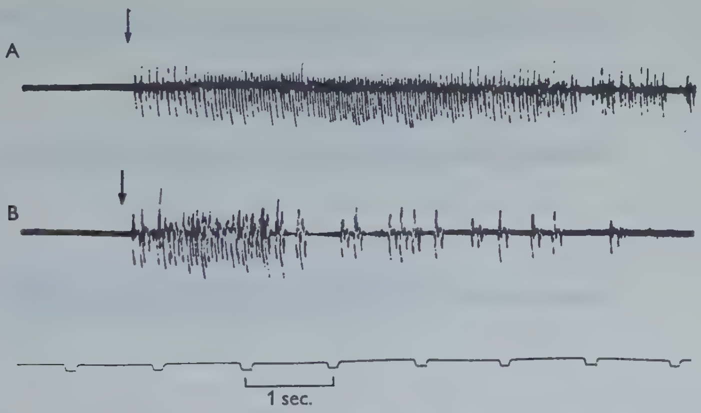

Fig. 2. (A) The effect of cooling the tarsus. At the arrow the temperature was dropped from 22° to 2° C. (B) The effect of warming the tarsus. At the arrow the temperature was raised from 2° to 22° C. The response was less that that shown in Fig. 2A even though carried out on the same tarsus.

activity. In general we found that over the range 0–25° C. the preparations were more sensitive to cooling than to warming. Preparations maintained below 13° C. were often found to be sensitive to a fall of 1° C., but most preparations required a rise of 5° C. before they became active. It was possible to obtain discharges as a result of increases of 1° C. but this only occurred at high temperatures, i.e. above 30° C. In most cases if we raised the temperature from 10° to 14° C. there was no activity—in fact if the preparation had been discharging as a result of a previous cooling, then warming stopped the discharge.

Preparations have been found which discharged for up to 7 min. after the tarsus had been cooled. The extent and duration of the discharge depended upon the extent to which the temperature was lowered. This is demonstrated in Fig. 3.

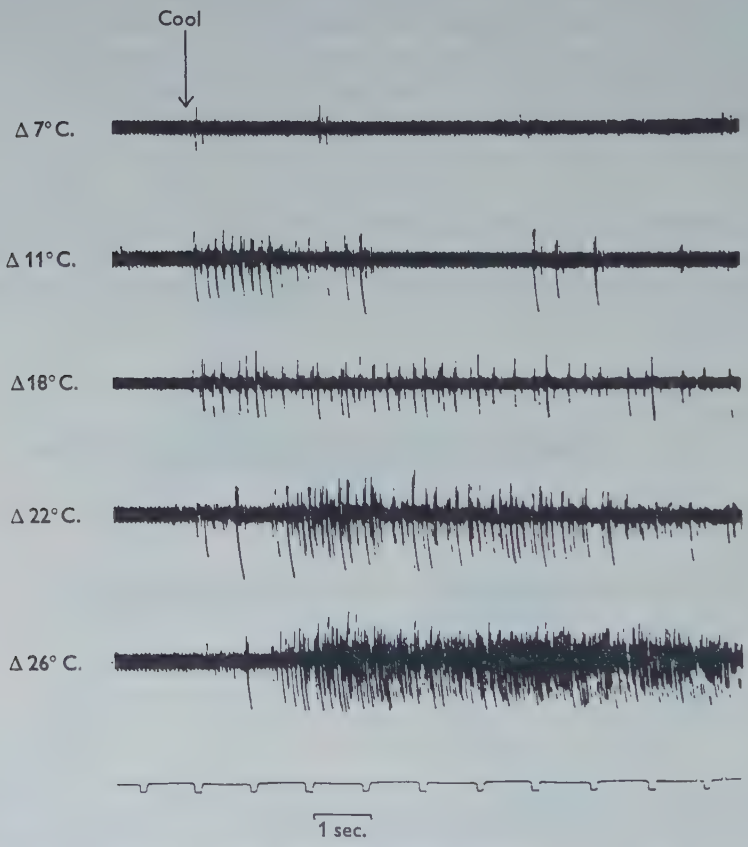

Fig. 3. The effect of cooling the tarsus. The tarsus was acclimatized to 22° C. and then the temperature was lowered by the amount shown to the side of the record. The activity was increased as the temperature drop increased.

Here the preparation was adjusted to 22° C. and the temperature then lowered. The activity of the preparation was recorded and the temperature then returned to 22° C. for 3 min. The preparation was then cooled once more and then returned to 22° C. In this way the sensitivity of the preparation to different changes of temperature was determined. A fall of 7° C. brought about only slight activity, whilst bigger temperature changes (Δ 11–26° C.) brought about greater activity which also lasted for a longer time.

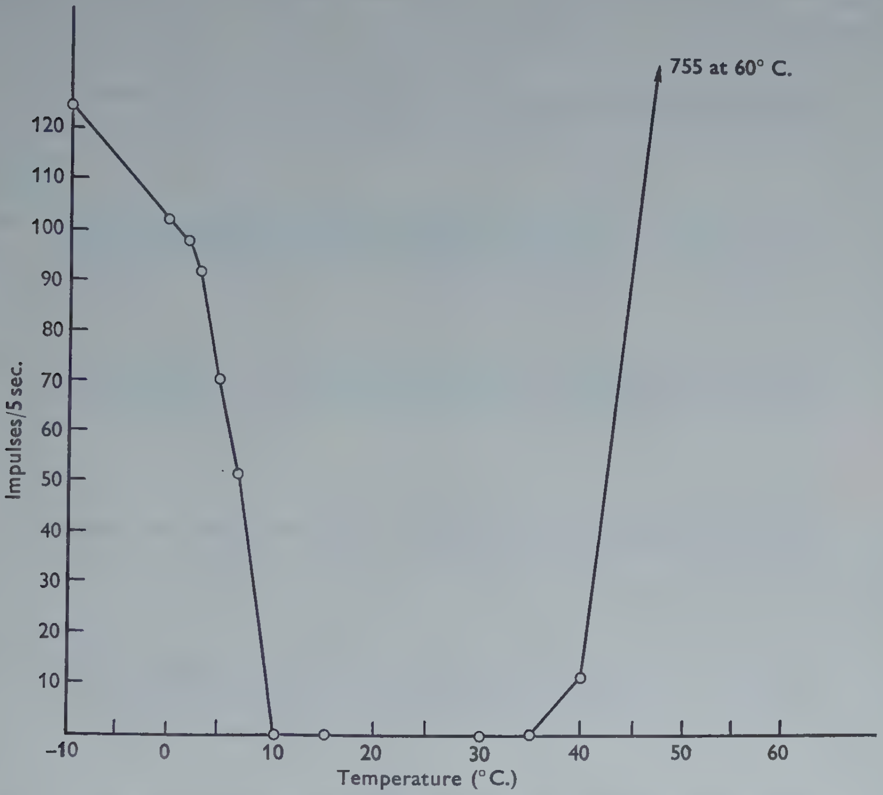

Fig. 4. The effect of temperature changes on the tarsus. The preparation was acclimatized to 22° C. and then subjected to various temperature changes. The activity is shown in this figure at different temperatures. It will be seen that the tarsus becomes more active when it is cooled below 10° C. and warmed above 35° C.

The sensitivity of the preparations varied slightly from leg to leg and from one region to another of the temperature range over which they were tested. In the experiment illustrated in Fig. 4 there was no activity till the temperature was dropped below 10° C. This preparation became active only above 35° C., a change from 22° to 30° C. having no effect. At the same time it should be noted that there was often a response when it was warmed from 10° to 22° C.; but in this experiment only changes from 22° C. are considered.

Cooling below 22° C. in general increased the activity, and in most cases if the preparation was warmed whilst the cold discharges were active, the activity then stopped. In some cases, however, warming brought about a sudden increase in activity for a few seconds before it faded away and stopped.

At times the preparations showed some continuous background activity due to the stimulation of proprioceptors and mechanoreceptors. In such preparations lowering the temperature brought in the activity of the cold receptor. This is shown in Fig. 5, where at the arrow the temperature was lowered from 22° to 5° C. At this point large impulses appeared which were easily distinguished from the background activity.

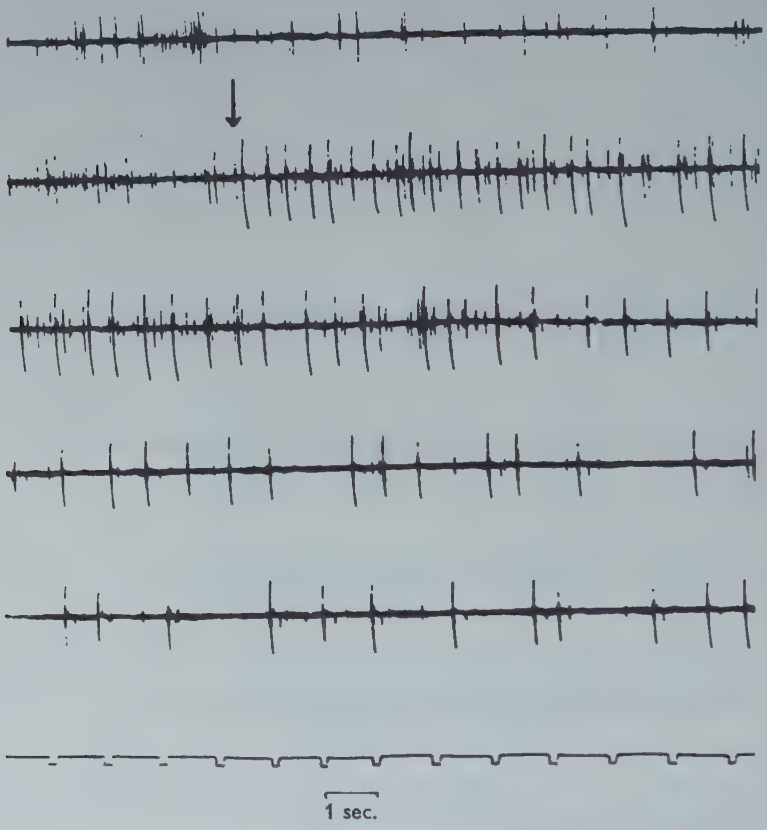

Fig. 5. The effect of cooling a leg showing background activity. There was electrical activity in the leg due to stimulation of a proprioceptor. At the arrow the temperature was lowered from 22° to 5° C. The cooling brought in a series of large potentials easily distinguished from the proprioceptive activity.

LOCALIZATION OF THE RECEPTOR WITHIN THE TARSUS

Since the receptor is active when only the pre-tarsus and claw are cooled, it would appear that this is the site of the cold receptor. In addition, it is this part that is raised from the ground when the animal is placed on a very cold surface. The site of the receptor was further localized by placing only part of the limb on the metal surface. There was no response when either the tip of the claw or the side of the claw were cooled. It was necessary for the pad (arolium) to be in contact with the metal before any response could be elicited. Other sensitive sites were found at the base of the first, second, third and fourth tarsal segments (unstippled in Fig. 1).

These are the regions that are unsclerotized and form the tarsal pulvilli. Local application of a cold copper rod confirmed that the arolium and pulvilli were the most sensitive parts of the tarsus. If these were amputated, a response was still obtained to cooling but the potentials were reduced in size. Similarly, if the cut stump of the tibia was cooled it evoked potentials which were, in general, smaller than those found after cooling the intact tarsus.

Gentle pressure on the pulvilli with a warm rod (20° C.) evoked a series of small potentials, but if the rod was cold (0° C.) the potentials were much larger in size. It is possible that the pulvilli have receptors sensitive to both touch and temperature and that cooling stimulated many receptors at the same time and hence brought about large potentials. On the other hand, there may be two types of receptors, one for touch and another for temperature. Histological studies are now being carried out to determine whether there is more than one type of sense organ on the pulvilli. There is some indication that the tactile receptors can respond to temperature since the hairs on the ventral surface of the first tarsal segment can be stimulated when a cold rod (−20° C.) is brought within 1 mm. of them; no contact being made in this case.

DISCUSSION

The literature on the occurrence and properties of temperature receptors in insects has recently been reviewed by Herter (1953). Most of the previous investigators have been concerned with the reactions of insects to heat and the way in which their behaviour alters if various parts of the body are removed. Herter (1924) brought a hot needle towards various parts of an insect's body and noted how near the needle had to be before the insect showed a response. The most sensitive part of *Blabera fusca* proved the labial palps which were sensitive when the needle was some $2\frac{1}{2}$–3 mm. away. The tarsi were sensitive when the needle was 0·5–1 mm. away. In *Liogryllus campestris* he showed the relative temperature sensitivity was, in order of decreasing sensitivity, labial palps, antennae, cerci and tarsi. Cappe de Baillon (1932) showed the presence of a heat receptor in the antennae of the phasmid *Menexenus semiarmatus* by removing parts of the antennae and finding when the animal lost its capacity to respond to localized heat. He showed that there was a receptor in the dorsal surface of the 12th antennal segment. Similarly, Herter (1939) and Kalmus (1948) showed a temperature receptor in the 14th segment of *Carausius morosus*. Wigglesworth & Gillett (1934*a*, *b*) showed that in *Rhodnius prolixus* the long hairs at the tip of the antennae were most probably temperature receptors. Slifer (1951) showed by extirpation experiments, and by noting the way in which normal and operated animals responded to a hot glass rod, that the antennal crescents and the body fenestrae were temperature receptors in *Locusta migratoria migratorioides*.

There seems to be very little information concerning either the electrophysiology of the temperature receptors, or the existence of a cold receptor. Barber (1956) has described the electrical reactions of a temperature receptor in the gnathobase of *Limulus*, and Hodgson & Roeder (1956) have shown that the sensory discharges from the taste receptors in *Phormia* are sensitive to temperature change, but in both cases only a positive response to an increase in temperature has been described. In neither case does the activity increase when the temperature is decreased. The receptors described here for the tarsus of the cockroach behaves more like the cold receptors described by Dodt & Zotterman (1952) in the cat's tongue. These receptors become more active when the temperature is lowered. The activity is proportional to the extent to which the temperature is lowered. The activity can last for some time after the receptor has been cooled, in some cases for 7 min. It is interesting to compare the response of the tarsal receptor with the responses found to temperature changes in the central nervous system of the cockroach. In the central nervous system there are several patterns of cell behaviour, some of which are identical with that described for the tarsal receptor. These cells only become active at low temperatures and their activity disappears on warming to approximately 13–28° C. There are other cells in the central nervous system which for a short time become more active when the temperature is lowered, but within a few seconds their activity is considerably reduced (Kerkut & Taylor, 1956).

It is difficult to tell if there are warm receptors in the tarsus. There are two distinct conditions under which warming the tarsus can lead to electrical activity. In the first case the tarsus responds when it is warmed 5° C. in the range from 0° to 28° C., but the activity lasts for a short time only. In the second case, the tarsus responds when it is warmed 1° C. above 30° C. In this case the activity is prolonged and may show definite bursts of injury potentials. Here the tarsus resembles the cold receptors described by Dodt & Zotterman (1952) in the cat's tongue, which also showed an anomalous cold discharge at higher temperatures.

SUMMARY

1. This paper describes the electrical responses of the isolated limb of the cockroach following localized warming and cooling.

2. There is a temperature-sensitive region in the pad between the claws, and in the first, second, third and fourth tarsal segments (the arolium and the pulvilli).

3. The preparation becomes more active when the temperature is lowered below 13° C. The activity increases as the temperature drop increases. It thus behaves like a cold receptor.

4. The preparation is sensitive to a drop in temperature of 1° C. over the critical range below 13° C.

5. The preparation is stimulated by warming it approximately 5° C. in the range 0–28° C., but the activity lasts for only a short time.

6. Warming the preparation above 30° C. brings about considerable prolonged electrical activity.

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EFFECTS OF HIGH HYDROSTATIC PRESSURES ON THE ACTIVITY OF FLAGELLATES AND CILIATES

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INTRODUCTION

Within the range of from 100 to 1000 atm. (about 1470–14,700 p.s.i.) high hydrostatic pressures affect living cells in various ways and in general adversely. Certain particular metabolic processes are known to be depressed; for instance, fermentation in yeast and (at ordinary temperatures) luminescence in bacteria. Various contractile processes associated wth protoplasmic gelation are inhibited; for instance, amoeboid movement, cleavage of eggs, and contraction of chromatophores. Certain biological activities, including various aspects of the response of nerve and muscle, are known to be enhanced at the lower end of this range of pressures; but at higher pressures activity is depressed or abolished. Aquatic animals are often stimulated to greater activity by pressures of about 100 atm., but they are paralysed or killed by pressures of from several hundred to 1000 atm.

The effects of high pressure are interpreted by Johnson, Eyring & Polissar (1954, with bibliography) in terms of the volume changes accompanying chemical reactions. The rate of a chemical reaction will be increased or decreased by high pressure according as to whether the formation of activated complex involves a decrease or increase in volume, and its equilibrium will be shifted in the direction of decreased volume. Metabolic effects of pressure are readily interpreted in terms of changed reaction rates. The effects on protoplasmic gelation are attributed by these authors to a shift in an equilibrium between extended and contracted protein molecules—an explanation which harmonizes well with observations on the antagonistic action of pressure and temperature. The effects of pressure on the numerous interacting metabolic processes of cells are likely to prove as complex as are those of temperature. There are also interesting possibilities of effects on cell structure which might affect the interactions of enzymes with substrates. Although the effects of pressure on luminescence of intact bacteria and of cell-free extracts show remarkable similarities, there are certain differences which may be due to structural organization (Strehler & Johnson, 1954).

The improved optical arrangements and the optical adaptability of the pressure vessel already described (Kitching, 1954) makes possible an investigation of the effects of pressure on the structure and activity of many different types of cell. This study of the effects of high pressures on Protozoa should provide an introduction to further detailed work. The fundamental interest of high pressure as a tool for the investigation of living reactions is clear enough. Moreover, even before interpretation of results is possible in biochemical terms, experiments with high pressures are likely to lead to a variety of unexpected observations, as already found with Suctoria (Kitching, 1954). The occurrence of bacteria and of various invertebrate animals in the ocean deeps suggests an inquiry into the nature of biochemical adaptation to these pressures and into the limits of acclimatization possible without genic mutation.

METHODS

Protozoa of the various species mentioned later were mounted in a hanging drop of their own culture medium on the upper window of the pressure vessel already described (Kitching, 1954). The dead space was completely filled with a mineral oil, usually Boots's medicinal paraffin B.P., but in some experiments an I.C.I. high boiling-point paraffin. There was no difference between the results obtained with these two mineral oils. Specimens left mounted in the vessel after treatment survived for a number of days. For some of the experiments a new vessel was used having a thermocouple junction within it (and also provided with channels in the wall for temperature control and with a lever passing through the wall). The experimental procedure was varied to suit the material and circumstances, but certain standard procedures were used for interspecific comparisons. Experiments are summarized under the 'Results' for each species in the following terms:

(A) Single treatment: a single period of treatment at a constant high pressure, preceded and followed by observations at no applied pressure (='zero pressure').

(B) Multiple treatment: more than one period of treatment at constant high pressure. These experiments are subdivided as follows:

(i) Standard step-wise: the pressure was raised by steps of 1000 p.s.i. (68 atm.), with no intervening return to zero; 5 min. were spent at each step. The pressure of the highest step depended on the requirements of the experiment and is stated. From this there was a direct return to zero pressure in 1–5 sec.

(ii) Direct: the pressure was raised directly from zero to any given pressure, and was always returned to zero between periods of treatment. There was no standard time of treatment or standard series of pressures.

(iii) Irregular: the arrangement of treatments did not conform to either of the types described above.

Both in direct and in irregular multiple treatment experiments, treatments liable to cause obvious damage were left until the end.

The limits of room temperature for each series of experiments are stated under 'Results'. For any one experiment this temperature did not fluctuate by more than ±0·5° C., except during overnight recovery periods. The light was fitted with a heat-absorbing filter. Ciliary frequency was measured stroboscopically in a number of experiments.

Compression of medicinal paraffin leads to an appreciable rise of temperature. In the new vessel a rise of 0·6–0·8° C. was registered on compression to 1000 p.s.i.; and the true value may have been greater. However, the temperature fell to within 0·2–0·3° C. of its original value in 30 sec., and within 2 min. the disturbance was no longer measurable. It is likely that the changes were similar in the old vessel (no. 1), as the internal cavity is similar in the two vessels and small compared with the bulk of surrounding steel. In certain experiments carried out with the new vessel, in order to limit the momentary change of temperature to a negligible value, the pressure was raised or lowered in substages of 250 p.s.i. or 125 p.s.i. every half minute ('subdivided pressure change') until the desired change had been accomplished. In some other cases pressure was released continuously and very slowly, for the same purpose; details are given under 'Results'.

Polytoma uvella

Culture: axenic, in Thorpe's (1932) medium.

Experiments: six standard stepwise (max. 14,000–18,000 p.s.i.), two single treatment, four multiple treatment (direct or irregular) (max. 3000 p.s.i.), very many individuals, 17·1–20·3° C.

In stepwise experiments, application of 1000 p.s.i. appeared to stimulate into activity such animals as were sluggish or stationary. Apart from this there was no noticeable effect up to 8000 p.s.i. From 9000 p.s.i. upwards the speed of swimming decreased, a few stopped movement at 10,000 p.s.i., and all eventually stopped at 14,000 p.s.i. The pressure required to stop movement was the same for a 4-day-old and a 33-day-old culture in adjacent hanging drops. At pressures of 16,000–18,000 p.s.i. some individuals developed what appeared to be minute blebs at the base of the flagella, but interpretation was difficult.

A few started moving again, slowly or very slowly, within about half a minute on release of pressure from 14,000 p.s.i. Most remained stationary for many hours, but had resumed normal activity by the next morning; in one experiment they were stimulated to activity sooner by the application of a moderate pressure (3500 p.s.i.). After stepwise experiments to 17,000 and 18,000 p.s.i., some few individuals (out of hundreds) eventually made slight wobbling movements, and a very few in each case swam again normally within 1–2 days of release of pressure.

The stimulating effect of moderate pressure was confirmed in single and multiple treatment experiments to 1000, 2000 and 3000 p.s.i. Without applied pressure about 25% of the individuals, as judged at a glance, were stationary and resting at the surface of the glass window. Most of these became active when the pressure was raised to 1000 p.s.i., and almost all were active at 2000 or 3000 p.s.i. On release of pressure, the number of stationary individuals had risen within a minute or two to about the same as before treatment. In order to test whether these results were caused by the change of temperature which accompanies compression or decompression, two tests were carried out with compression by subdivided pressure change (125 p.s.i. per half minute) to 2000 and 3000 p.s.i., and with continuous slow release to zero pressure in 34 and 56 min. The same changes in activity occurred.

Chlamydomonas pulsatilla

Culture: tap water with soil and trace of NH_4MgPO_4.

Experiments: two standard stepwise, maximum 13,000 p.s.i., very many individuals, 20·0–20·1° C.

Swimming continued actively up to 7000 or 8000 p.s.i., but became noticeably and progressively slower from 9000 p.s.i. upwards. Some individuals stopped altogether at 11,000 p.s.i., more still at 12,000 p.s.i., and all at 13,000 p.s.i. On release of pressure all remained motionless at first, but a few individuals started making small movements within about an hour. By next morning (18–24 hr. after release of pressure) less than a quarter of the individuals were moving nearly normally, while the rest were motionless and probably dead.

Astasia longa

Culture: axenic, in Pringsheim's (1946, p. 47) yeast extract–acetate–peptone medium. Experiments: five standard stepwise + one similar but with stepwise release also, max. 9000 or 10,000 p.s.i., very many individuals, 12·2–14·0° C.

The organisms swam actively and with little or no loss of speed at pressures up to 4000 p.s.i. They lost speed progressively from 5000 p.s.i. upwards; at 8000 p.s.i. many were stationary and the rest were very slow; and at 9000 p.s.i. or 10,000 p.s.i. most became motionless, and movement in the rest was reduced to a faint wobble. The body was distinctly shorter, but still spindle-shaped, at 8000 p.s.i., but in most cases rounded or irregular at 9000 p.s.i.; the wobbling at 9000 p.s.i. was confined to those few which still remained spindle-shaped. On release of pressure to zero, nearly all started moving immediately, but the movement of those in which the body had become rounded up was in circles. Within half an hour after release of pressure many had resumed their usual elongated shape and their normal swimming speed. With stepwise release of pressure some movement was restored at 8000 p.s.i., and there was active swimming movement at 7000 p.s.i. down to 1000 p.s.i. However, the body remained rounded, pear-shaped, or irregular, until after all pressure had been released.

Paramecium spp.

Culture: Bristol tap water and carrot extract.

(*a*) P. aurelia, *effects of pressures of up to* 1000 *p.s.i.*

Experiments: six multiple treatment direct, treatments of 2–10 min., numerous individuals, 16·7–21·3° C.; three additional slow-release experiments, see below.

The organisms became noticeably less active with increasing pressure, with a tendency to halt among the debris or at the edge of the drop. This decrease in activity was noticeable at 500 p.s.i. and doubtfully so at 200 p.s.i. There was an immediate return to full activity on release of pressure, noticeable after pressures of 200 p.s.i. or over. The immediate response was one or more turns, followed by active swimming.

Three experiments were carried out with slow release of pressure, to eliminate effects of temperature change. Release was from 1000 p.s.i. to zero, in 18, 10 and 22 min. All individuals were swimming actively at zero pressure before treatment and became slow or stationary at 1000 p.s.i. During release there was a gradual increase in the number swimming actively until, at zero or above, all were fully active.

(*b*) *Comparison of* P. aurelia *and* P. caudatum *in stepwise experiments*

Experiments: six stepwise, max. 4000–7000 p.s.i., numerous individuals, 17·6–20·5° C.; in three of the experiments each species was mounted in a separate drop, side by side, and in three the cultures were mixed in equal quantities.

P. aurelia became slow or stationary at 1000 p.s.i., although a few continued to move slowly at 2000–4000 p.s.i. Some began to swim and finally to rotate on the long axis at 6000 p.s.i. Many rotated violently and then became distorted and irregular in shape at 7000 p.s.i. On release of pressure, those least affected immediately swam rapidly. The distorted ones remained stationary, apparently dead. Rise of pressure to 5000 p.s.i. in one step halted some but not all. Swimming was resumed on release of pressure, and there were no other obvious effects.

P. caudatum swam actively before treatment and at 1000 p.s.i., more slowly at 2000 and 3000 p.s.i. Some developed blisters at 4000 p.s.i.; all developed blisters by 5000 or 6000 p.s.i., but some continued to swim even so. Most were stationary at 7000 p.s.i. On release of pressure from 5000 and 6000 p.s.i., there was immediate swimming movement, in spite of blisters, but most became quiet later. On release from 7000 p.s.i. there was no recovery of movement. When the pressure was raised to 5000 p.s.i. in one step, all immediately shortened, underwent rapid rotation on the long axis, and became very blistered. On release of pressure (after 2 min.) there was at first some slow movement, but all came to a halt within 15 min. The body shape was gradually restored, and the surface cilia were observed to beat.

(*c*) P. caudatum, *contractile vacuole*

Experiments: one single treatment, two multiple treatment direct (periods of up to 18 min. at pressure), one multiple treatment irregular, 11·7–14·0° C. A single *Paramecium* was confined in a volume sufficiently thin to impede rotation about the long axis, but sufficiently thick and extensive to allow considerable movement in a horizontal plane. The anterior contractile vacuole was observed, timed, and measured.

The rate of output of the contractile vacuole was reduced by increase of pressure (Fig. 1), and vacuolar activity was halted at 5000 p.s.i. The effect of pressure was mainly on the vacuolar duration, rather than on ultimate diameter. On release of the pressure the contractile vacuole resumed its normal rate of output, or even exceeded this for a time, provided that the treatment had not been too drastic. The effect of pressure on vacuolar output is summarized in Fig. 2.

Tetrahymena pyriformis

Culture: axenic in beef extract (Lab Lemco) peptone medium.

Experiments: nine standard stepwise, max. 11,000–16,000 p.s.i.; three single treatment to 16,000 p.s.i. for 5, 10 and 15 min.; two multiple treatment direct; very many individuals; 16·2–20·2° C.

In stepwise experiments, swimming continued very actively to about 7000 p.s.i., and at this pressure those animals which had previously moved slowly along the glass/water interface swam freely and fast. At higher pressures swimming became progressively slower. Some movement continued up to 11,000 p.s.i., but all were stationary by the end of the period at this pressure or early during the period at 12,000 p.s.i. At the higher pressures, and especially from 12,000 p.s.i. upwards, some individuals became bulbously deformed and finally spherical, but many remained normal in shape or nearly so even at 16,000 p.s.i. In one standard stepwise experiment 4-day-old and 48-day-old cultures were mounted in adjacent hanging drops for simultaneous observation. Effects on the two cultures were very similar; the older culture was possibly the less active at the higher pressures, but showed some slight movement at 10,000 p.s.i.

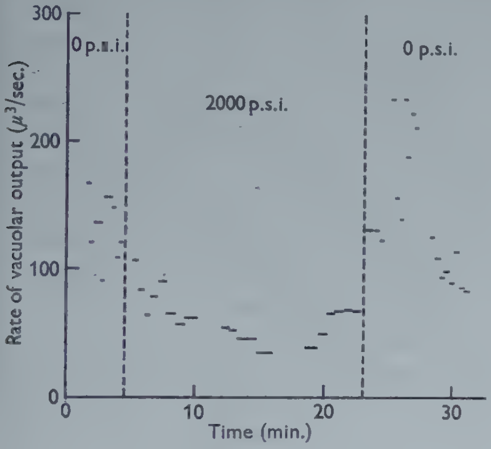

Fig. 1

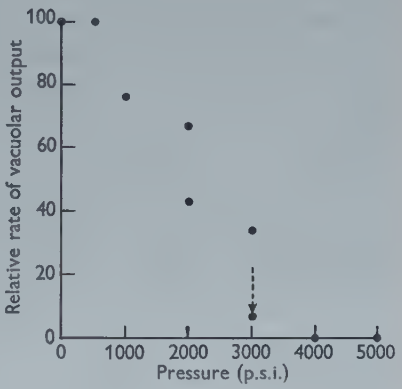

Fig. 2

Fig. 1. Effect of hydrostatic pressure of 2000 p.s.i. (136 atm.) on the rate of output of the anterior contractile vacuole of *Paramecium caudatum*.

Fig. 2. Relation between hydrostatic pressure and rate of output of anterior contractile vacuole in *Paramecium caudatum*.

On release of pressure from 11,000 p.s.i., in some cases there was a resumption of slow movement in about 5 sec., but most individuals took up to 20 min. to recover moderate swimming speed. After release from higher pressures (14,000–16,000 p.s.i.), the organisms remained stationary. The contractile vacuole reappeared as one or more vacuoles at the posterior end of the organism, and often grew to a considerable size before discharging. In many cases the organisms swelled and became spherical, a thin layer of protoplasm surrounding a greatly enlarged vacuole. In a few cases after 14,000 p.s.i., in many after 15,000 p.s.i., and in all after 16,000 p.s.i., the organisms burst and disintegrated. Slight oscillatory movements were noted in some individuals 6 hr. after release from 15,000 p.s.i. and half an hour after release from 14,000 p.s.i. Many eventually recovered and divisions were seen on the following day in both cases.

In three experiments many individuals of *T. pyriformis* were exposed directly to 16,000 p.s.i. (for 5, 10 and 15 min.) and then returned to zero pressure. All stopped movement immediately at high pressure, and many became spherical. After release from a 5 min. exposure, the stages were as described above; some eventually burst, but many recovered and divisions occurred by the next day. After 10 min. exposure only one recovered out of the many used in the experiment, although this one subsequently divided many times; none recovered after 15 min. exposure. Thus a 10 min. single treatment achieved practically the full mortality obtained by standard stepwise procedure to the same pressure.

Holophrya sp.

Culture: Miquel sea water (Allen & Nelson, 1908, p. 428) with growing diatoms.

Experiments: four standard stepwise, max. 12,000–14,000 p.s.i., numerous individuals, 16·4–19·0° C.

Before application of pressure most of the organisms were stationary and feeding, especially among debris, although some short excursions were made from time to time. They became slightly more restless at pressures up to 4000 p.s.i., and very distinctly so at 5000–7000 p.s.i., especially immediately after a rise in pressure. Many swam continuously at 8000 p.s.i. and all did so at 9000 p.s.i. At 10,000 p.s.i. swimming was at first fast, in spirals, but became slower. All, or almost all, stopped by the end of the period at 12,000 p.s.i. One individual moved very slowly at 13,000 p.s.i., but finally stopped. Many became rounded at 12,000 p.s.i., and all were rounded at 13,000 p.s.i. and 14,000 p.s.i.

A few began to move slowly within 1 min. after release from 12,000 p.s.i., and all appeared normal by the next day (20 hr.). All developed large vacuoles (presumably contractile vacuoles) in 10–15 min. after release from 13,000 p.s.i.; many recovered within 2 days and appeared normal again. There was no sign of life nor any recovery after release from 14,000 p.s.i.

Colpidium campylum

Culture: Bristol tap water + carrot extract.

Experiments: four standard stepwise, max. 12,000 p.s.i. (three experiments) and 15,000 p.s.i., very many individuals, 17·2–22·6° C.

At 1000–3000 p.s.i. the number of individuals which were stationary alongside debris increased, in two of the experiments very greatly; but at still higher pressures more became free-swimming. At 6000 p.s.i. the rate of swimming was distinctly slower, and it became slower still as pressure was increased. At 9000 p.s.i. a small number had stopped and all came to a halt before the end of the period at 12,000 p.s.i. Many were slightly more rounded at 10,000 p.s.i., and all were spherical or nearly so at 14,000 p.s.i. Many cytolysed at 15,000 p.s.i.

On release of pressure from 12,000 p.s.i. all remained stationary at first, but within a few minutes a number were seen to be making exceedingly minute backwards and forwards oscillations. Normal swimming was resumed in some individuals in about 10 min., in others after a period ranging up to several hours. On release from 15,000 p.s.i., in a few which had not cytolysed a contractile vacuole began to operate, but all soon disintegrated.

Colpoda cucullus

Culture: Bristol tap water + carrot extract.

Experiments: nine standard stepwise, max. 6000–8000 p.s.i.; two control (see below); numerous individuals, 17·2–21·2° C.

At no applied pressure most were stationary among debris, or creeping among it, but a few were sometimes free-swimming. At 1000 to 3000 p.s.i. there was an increasing restlessness among thigmotactic individuals, until by 3000 p.s.i. all were swimming freely. With each increase in pressure over this range, the restlessness did not develop or increase instantly, but took about half a minute or longer. At 2000–3000 p.s.i. there was a tendency to swim for brief periods rapidly in close circles, alternating with periods of normal swimming. Swimming was distinctly slower at 4000 p.s.i., and movement ceased at 6000 or 7000 p.s.i., more usually the former. Stopped individuals often became blistered or distorted, and the contractile vacuole was abnormally large and failed to discharge.

On release of pressure the organisms all remained stationary. In most cases the contractile vacuole discharged within about a minute, and after about 20 min. the normal body shape had been restored, with deeply incurved pharynx. Within 2–8 hr. of release of pressure many but not all individuals formed thin-walled resting cysts, from which they usually emerged within a few hours. Excysted individuals displayed normal activity. In two experiments a few division cysts were also formed. In control experiments, in which the organisms were mounted as usual in the pressure vessel but without subjection to high pressure, a very few division cysts were formed during the 8–9 hr. period of observation, but no resting cysts. More experiments are required with more uniform material before conclusions can be drawn about induction of encystment by treatment with high pressure.

In other experiments, individuals subjected to pressures of 7000–9000 p.s.i. disintegrated suddenly.

Spirostomum ambiguum

Culture: Chalkley's solution with wheat grains, in tall jar.

Experiments: three standard stepwise, max. 6000, 10,000, and 11,000 p.s.i., numerous individuals, 14·2–16·5° C.; three multiple treatment direct, with measurement of swimming speed, single individuals, 14·2–17·1° C.; two stepwise releases of pressure (see below), 22·5° C.

(*a*) *Swimming speed and body shape*

The average swimming speed declined (reversibly) from 2000 p.s.i. upwards; a few organisms ceased to make any progress at 5000 p.s.i., but a few continued up to 10,000 p.s.i. Some very weak ciliary activity was observed at 10,000 and 11,000 p.s.i. From 6000 p.s.i. upwards, a number of individuals became bent into a U and swam in circles. A number cytolysed at 9000–10,000 p.s.i. On release of pressure, those which had not cytolysed began to move again quite briskly, although they were contorted and deformed. Some broken-off pieces of *Spirostomum* also moved again actively. In another similar experiment (see below) one specimen continued to move slowly at 11,000 p.s.i., and some others recovered activity and approximately normal shape over a period of 22 hr. on release of pressure.

During a 12 min. exposure to 8000 p.s.i., the numerous individuals present became distorted by a shrinkage of the anterior end, most of the protoplasm collecting in the rest of the body. The anterior end bent around on itself and the *Spirostomum* swam in circles or spirals. Finally each became shaped like a sphere with a tail attached, and portions of the latter broke off with the cilia still beating rather weakly. On release of pressure there was some immediate recovery of movement, and distorted hulks of *Spirostomum* gyrated with actively beating cilia. A few small fragments showed some activity.

(*b*) *Behaviour*

It was noticeable in stepwise experiments that there was no reversal or avoiding reaction at the higher pressures. At 3000 p.s.i. the avoiding reaction appeared normal and the organisms were distributed throughout the drop; at 4000 p.s.i. and over no avoiding reaction was seen, and the organisms swam steadily around the edge of the drop but were absent from the middle.

Release of pressure from 6000 p.s.i., 4000 p.s.i., or 2000 p.s.i., to zero was immediately followed by a period of backwards swimming, lasting for about a minute after 6000 p.s.i. and less time after the lower pressures. Release of pressure was also carried out from 6000 p.s.i. by 1000 p.s.i. steps with 5 min. at each (one experiment). On release from 6000 p.s.i. to 5000 p.s.i. organisms continued swimming at the edge of the drop, and there was no reversal. A few cases of brief reversal were seen at 4000 p.s.i.; at 3000 p.s.i. there were a number of short reversals; and on release to 2000 p.s.i. many organisms immediately reversed, and many left the edge of the drop. Behaviour appeared normal at 2000, 1000 and 0 p.s.i. After recompression, and on release from 8000 p.s.i. by 2000 p.s.i. steps of 5 min. each, there was no reversal at 6000 p.s.i. and very little at 4000 p.s.i.; many reversed immediately on release to 2000 p.s.i. and to 0 p.s.i., and at these pressures the normal avoiding reaction was resumed and the organisms became distributed throughout the drop.

Stentor polymorphus

Culture: Chalkley's solution with wheat grains.

Experiments: eight standard stepwise + four similar but with subdivided pressure change and stepwise release, max. 8000–11,000 p.s.i., few or single individuals; ten multiple treatment irregular experiments to lower pressures, for membranellar frequency, single individuals; 13·3–20·0° C.

At 1000 p.s.i. there was either no significant change or (usually) a slight decrease in frequency of beat of the adoral membranellae. At higher pressures the frequency decreased progressively (Fig. 3). In the range of 2000–3000 p.s.i. the fall in frequency took place at any rate within a few seconds of the application of pressure, and was reversed with equal rapidity when the pressure was released. At somewhat higher pressures, varying from 4000 to 7000 p.s.i., metachronal rhythm broke down; some of the membranellae stopped completely, and the beating of the remainder was reduced to a weak and irregular flicker of small amplitude. This sudden stoppage was often associated with a relaxation or outward bulging of the disk, the points of the membranellae being directed stiffly backwards. In one case flickering persisted even up to 10,000 p.s.i., but finally stopped. At 8000–10,000 p.s.i. the body became distorted and irregular in shape, with the foot-wall collapsing and the foot bulging locally. In some cases in standard stepwise experiments cytolysis occurred at 11,000 p.s.i., but a few individuals recovered after this pressure and appeared normal 16 hr. later. On return to no applied pressure the body contracted either suddenly or slowly. The membranellae resumed their activity, sometimes immediately, but this resumption was interrupted by contraction of the body when this occurred. The normal rate of beating was sometimes attained only after several minutes, or not at all after the higher pressures. Sometimes further sudden contractions of the body occurred, and were often followed by a temporary reduction in the frequency of membranellar beat.

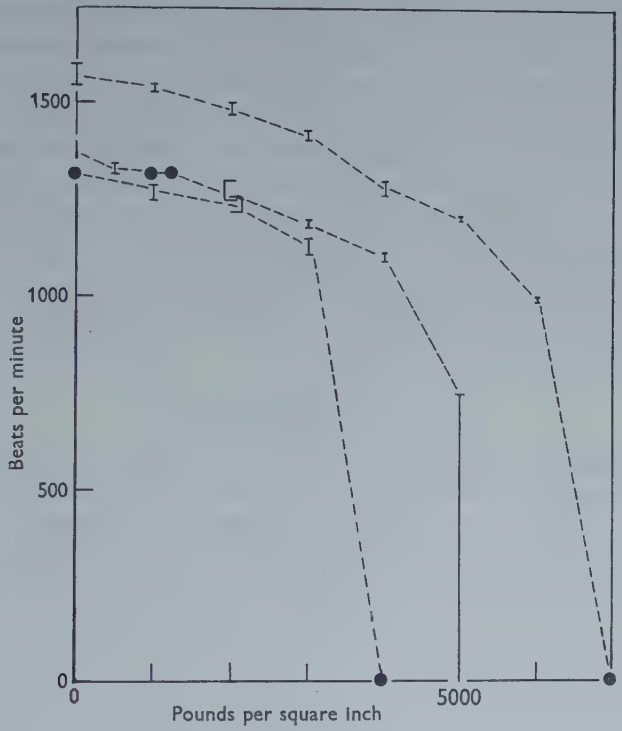

Fig. 3. Relation between hydrostatic pressure and frequency of the membranellar beat in *Stentor*. Results for three individuals and the limits of readings at each pressure are shown.

Stylonychia mytilus

Culture: Bristol tap water with wheat grains.

Experiments: two standard stepwise; one multiple treatment direct with same series of pressures, max. 7000 or 8000 p.s.i., one or two individuals, 16·5–17·7° C. (material limited).

Before treatment, at no applied pressure, the organisms crawled around, with occasional avoiding reactions, short darts forward, and brief periods of no movement. At 1000 and 2000 p.s.i. there was no progression (two experiments) or much less, and there was none at 3000–5000 p.s.i.; feeding currents, however, continued. Release of pressure restored normal activity immediately. At 4000 p.s.i., and more especially at 5000 p.s.i., the locomotory membranellae and cirri, previously motionless, began to flicker. At 6000–7000 p.s.i. all the cirri and membranellae beat wildly and the body turned in circles, but towards the end of the period at 7000 p.s.i. movement of cirri and body almost ceased. There was instant recovery of movement when the pressure was released, and the animals swam with an irregular rolling or circling motion. At 8000 p.s.i. there was some disintegration of the posterior end of the organism, with loss of cirri, but a considerable measure of normal activity was recovered by 3 hr. after release of pressure.

Euplotes sp. (*patella* group)

Culture: pond water + wheat grains.

Experiments: two standard stepwise, one multiple treatment direct, max. 10,000 or 11,000 p.s.i.; one multiple treatment irregular; numerous individuals, 15·9–20·6° C.

Before pressure was applied, the organisms were creeping among debris in short runs or sometimes swimming freely, with short alternating periods of no movement. At 1000 p.s.i. there was a just noticeable increase in activity, more time being spent in running or swimming and less at rest. At 2000–4000 p.s.i. there was continuous running, without any stops; on release of pressure intermittent stoppages recurred, as before treatment. At 5000–8000 p.s.i. the organisms swam actively in small circles; on release of pressure the organisms stopped swimming and spent most of the time stationary, with occasional short runs. At 9000–10,000 p.s.i. swimming was much weaker, and eventually ceased, the membranellae only moving very slowly. At 10,000 p.s.i. a group of vacuoles was seen in the posterior part of the body, as though contributory vacuoles had failed to fuse to form a contractile vacuole. Some individuals failed to recover on release of pressure from 10,000 p.s.i.; others instantly resumed swimming for a few seconds, and then returned to the normal behaviour of intermittent runs and rests.

Vorticella sp. ? *marina*

Source: River Avon, Bristol (brackish water).

Experiments: two standard stepwise; three multiple treatment direct, max. 6000 or 7000 p.s.i.; 15·9–20·1° C.

In all cases after an increase in pressure the ciliary frequency fell during the first minute or two to a lower level. At 4000 p.s.i. and over there was a slow downward drift throughout the 5 min. exposure, The cilia stopped at 6000 p.s.i. (one experiment) or 7000 p.s.i. (two experiments). On release of pressure the cilia always beat faster again and recovered their original frequency within a minute or two after a 5 min. exposure to 1000–3000 p.s.i. Recovery was slow after exposure to higher pressures.

During a prolonged exposure (1 hr.) to 8000 p.s.i. the contractile vacuole failed to discharge, but grew steadily larger until its diameter exceeded half the width of the body. Almost immediately on release of pressure it discharged, and thereafter operated normally.

Cothurnia ? *curvula*

Source: River Avon, Bristol (brackish water).

Experiments: two multiple treatment direct, single individuals, 15·5–17·8° C.

There was no significant difference in frequency of discharge, ultimate diameter, or rate of output of the contractile vacuole at 1000 or 2000 p.s.i., but variation was considerable, so that minor differences could not have been detected. At higher pressures the ultimate diameter and rate of vacuolar output were reduced; vacuolar activity was very low at 5000 p.s.i. and reduced to zero at 7000 p.s.i.

Epistilis plicatilis

Source: shells of freshwater snail *Planorbis* sp. from pond.

Experiments: four multiple treatment irregular; observations on single zooid of colony, 19·0–21·0° C.

In all four experiments an increase of pressure within the range 1000–3000 p.s.i. produced an immediate significant increase in frequency of ciliary beat, and this effect was immediately reversed on release of pressure. At 1000 p.s.i. the ciliary frequency was increased by 3, 7, and 7%; at 2000 p.s.i. by 6, 8 and 9%; and at 3000 p.s.i. by 4, 5 and 5%, as compared with the frequency with no applied pressure. (In one case, illustrated in Fig. 4, contractions of the body prevented any assessment of the results at 3000 p.s.i.) At 4000–5000 p.s.i. and higher pressures there was a decrease in rate of beat, and the body also underwent repeated contraction. At 6000 p.s.i. the contractions were shorter in extent but they continued throughout the treatment; at 7000 p.s.i. they were reduced to short spasms, and at 8000 p.s.i. no contractions occurred. At 10,000 p.s.i. the body remained motionless, and there was no visible vacuolar activity. On release of pressure from 10,000 p.s.i. the cilia began flickering immediately, and the contractile vacuole resumed activity within a few minutes. The cilia subsequently resumed an active beat, although the frequency was still less than before treatment.

Carchesium polypinum

Source: a freshwater aquarium tank.

Experiments: one preliminary multiple treatment irregular, four single treatment (2000–8000 p.s.i. for 15–51 min.), 17·5–18·7° C., single zooid for contractile vacuole.

This organism contracted its body periodically, and this was particularly troublesome at moderate pressures (around 4000 p.s.i.). Contraction ceased after a few minutes at 6000 or 8000 p.s.i., and did not occur at 10,000 p.s.i. The ciliary beat was, of course, interrupted by the contraction, and did not in any case occur when contraction had ceased at the higher pressures. Ciliary beating was restarted within 2 min. of release of pressure after 5 min. exposure to 10,000 p.s.i., and within a few seconds or less after release from 6000 p.s.i. (30 min. exposure) or 8000 p.s.i. (51 min. exposure).

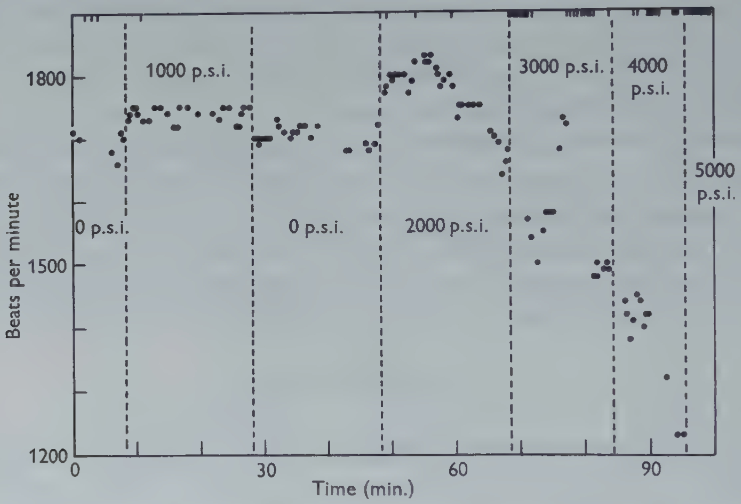

Fig. 4. Effects of hydrostatic pressure on ciliary frequency in *Epistilis plicatilis*. Each small vertical mark at the upper margin records a contraction of the body.

The frequency, ultimate diameter, and rate of output of the contractile vacuole were not significantly affected at 2000 p.s.i. Observations could not be made at 4250 p.s.i., owing to repeated contractions of the organisms. Vacuolar activity was abolished at 6000 and 8000 p.s.i., but the rate of output was exceptionally high immediately after release from these pressures.

DISCUSSION

The various flagellates and ciliates used in this work are very different morphologically, and it must be recognized that their mechanisms of activity and behaviour are also likely to be diverse. A ciliate such as *Paramecium* or *Spirostomum* may be said to show three levels of locomotor behaviour: (1) the simple beating of the various cilia and membranellae, which may vary in frequency, in amplitude, and in the direction of the effective stroke, (2) the co-ordination of the separate cilia in respect of these characteristics to give metachronal rhythm, acceleration, reversal, or any other co-ordinated activity, and (3) the super-co-ordination of these co-ordinated activities to give a response such as the avoiding reaction—reverse, turn and advance. Rather little is known about the operation of activities at these three levels. It has generally been held that metachronal rhythm involves the passage of a co-ordinative impulse along the ciliary row (Gray, 1928; Worley, 1934; Sleigh, 1957).

Transmission of this impulse probably involves a phase of excitation within the cilium (Bradfield, 1955) and a phase of conduction between adjacent cilia. It is not known to what extent the 'neurofibrillar' or 'neuromotor' system takes part in conduction between the cilia. Recent work on the fine structure of this system in *Paramecium* (Sedar & Porter, 1955) has shown that the kinetodesma is less continuous than had been supposed, but detailed information is wanted in many other types. The more condensed neuromotor system of hypotrichs has been regarded as a system for the co-ordination of certain of the cirri (Taylor, 1920). It is not known whether such co-ordination as may occur (either via the neuromotor system or otherwise) is operated by stimulation or inhibition. Various cilia can be reversed by a direct current, and there are observations connecting the direction of metachronal wave in *Opalina* with the potential difference across the cell surface (Kinosita, 1954). It is clear that any present attempt to explain the effects of pressure on behaviour must be speculative.

The frequency of ciliary beat in Protozoa is normally depressed by increase in pressure, at any rate at the temperatures used in these experiments. However, in *Epistilis plicatilis* there is an increase for pressures of up to 3000 p.s.i., but thereafter a decrease; and in *Stentor polymorphus* the frequency sometimes remains unchanged up to 1000 p.s.i. Of the various biological processes which are accelerated or enhanced by moderate pressures, bacterial luminescence has been investigated in detail. Pressure only increases luminescence at rather high temperatures, at which luminescence is less than normal. This increase is interpreted by Johnson *et al.* (1954) in terms of a shift in the equilibrium between an enzyme and its own denaturation product in the direction away from denaturation, while the decrease at still higher pressures is attributed to a direct effect of pressure on the rate of a chemical reaction. A similar type of explanation could be applied to the membranellar frequency of *Epistilis plicatilis*, although the true explanation may be more complicated.

The lateral cilia of *Mytilus* show a brief increase in frequency in reponse to the application of pressure (Pease & Kitching, 1939), and this overshoot effect is also interpreted by Johnson *et al.* in terms of chemical reaction rates. It is not surprising that different kinds of cilia from different animals should give different results; with a number of reactions involved the possibilities for variation in the pattern of response are considerable. *Paramecium* spp. is able to swim for a time after the removal of all oxygen (Kitching, 1939), although a sufficient hydrostatic pressure will immobilize it immediately: this suggests that pressure depresses at least one reaction (among others) which is more closely associated with the ciliary beat than those involving molecular oxygen.

Both flagella and cilia are capable of beating for short periods at pressures of up to 10,000–12,000 p.s.i., and in one case (*Polytoma uvella*) the flagella have shown

Table 1. *Cessation of movement and onset of structural damage in standard stepwise experiments* (p. 495)

Species	Pressures at which movement ceases (p.s.i.)	Pressures at which structural damage becomes evident (p.s.i.)	Nature of structural damage
Polytoma uvella	10,000–14,000	16,000–18,000	Minute blebs on some only
Chlamydomonas pulsatilla	11,000–13,000	—	None seen
Astasia longa	8,000–10,000	8,000–10,000	Became rounded or irregular in shape
Paramecium aurelia	7,000 or over	7,000 or over	Became distorted and irregular in shape
P. caudatum	4,000– 7,000	4,000–6,000	Became blistered
Tetrahymena pyriformis	*11,000–12,000	12,000 or over	Became bulbously deformed or rounded
Colpidium campylum	9,000–12,000	10,000–14,000	Became rounded or spherical
Colpoda cucullus	6,000– 7,000	6,000–7,000	Became distorted or blistered
Spirostomum ambiguum	10,000–11,000	6,000 and upwards	Became bent, contorted, or deformed
Stentor polymorphus	†9,000–10,000	8,000 and over	General distortion, especially bulging of foot; some bulging of disk at even lower pressures

* Or 9000 for those at edge of drop.
† Metachronal rhythm lost at 4000–7000 p.s.i.

some slight activity even up to 14,000 p.s.i. Stoppage usually occurs after treatment sufficient or nearly sufficient to cause some visible structural alteration or damage to the cell as a whole, such as rounding up, distortion, or blistering (Table 1). If a sufficient pressure is applied, there is an immediate slowing down or stoppage of ciliary beat; and if the pressure is released without undue delay, normal or nearly normal activity is immediately resumed. On the other hand, the effects of pressure on activity become more pronounced with the passage of time, and recovery takes longer after a longer treatment. Although the activity of flagella and cilia and the integrity of the cell structure are evidently correlated, it is not possible to say whether activity stops because of structural degeneration, or whether both these phenomena result from an unbalancing or inhibition of metabolic processes. The second hypothesis seems the more probable. Further, we may suggest that deformation or disintegration of the cell is assisted by the extra osmotic stress imposed by stoppage of the contractile vacuole, and also perhaps by unstabilizing effects of a breakdown of ionic regulation and by additional osmotic disturbances which are likely to result from metabolic failure.

There is one exception to the general correlation between structural integrity and continued ciliary activity. In *Spirostomum ambiguum* the cilia continue to beat even after the body has been drastically distorted. This ciliate is so large that a very limited solation of cortical gel may well result in rounding up or fragmentation, whereas in other smaller Protozoa distortion probably does not occur until the metabolic effects of high pressure have become acute. The bulging of *Stentor polymorphus* and the posterior disintegration of *Stylonychia mytilus* at high pressure also emphasize the skeletal importance of cortical gels in the larger ciliates.

In certain cases (*Polytoma uvella*, *Tetrahymena pyriformis*, *Holophrya* sp., *Colpoda cucullus* and *Euplotes* sp.) a moderate increase of pressure causes an increase in locomotor activity. This is particularly well illustrated by the flagellate *Polytoma uvella*, of which stationary individuals are caused to swim. There are also certain cases in which the application of moderate pressure decreases locomotor activity, although at still higher pressures the organisms may become more active again; this is true of *Colpidium campylum*, *Paramecium aurelia*, and *Stylonychia mytilus*. Various explanations might be offered for these effects. Pressure might operate an already existing kinesis normally brought into play by some different but related factor. For instance, a kinesis responsive either to metabolic conditions or to mechanical pressure might be called into play by hydrostatic compression. It may be relevant that *Polytoma uvella* in a hanging drop is also roused to activity if exposed to pure nitrogen. Alternatively, pressure might act specifically upon some cell component or system concerned with the mechanism of activity; this explanation might seem appropriate to *Stylonychia mytilus*.

It is particularly interesting that in certain cases co-ordination appears to break down before the capacity to beat is lost. The inhibition of the avoiding reaction in *Spirostomum ambiguum* at pressures of 4000 p.s.i. and over may be attributed to an inhibition of reversal, by which the whole reaction would be initiated. While unable to make the avoiding reaction, the organisms swim straight ahead, but when they reach the edge of the drop they are guided around by its curvature and cannot leave it. The wild beating of the membranellae of the hypotrich *Stylonychia mytilus* at high pressures could be ascribed either to uncontrolled stimulation or to a suppression of inhibition, but a study of the mechanism of behaviour of this organism is needed. It is possible that the breakdown of metachronal rhythm which occurs in *Stentor* at relatively low pressures is due to a failure of the stimulating mechanisms, perhaps associated with a structural change arising from cortical solation.

SUMMARY

1. The effects of brief exposure to high hydrostatic pressures, ranging in some cases up to 18,000 p.s.i. (1225 atm.) has been studied in various flagellates and ciliates. Closely related species may react very differently.

2. In general high pressure depresses flagellar or ciliary movement, and in most cases, under the standard conditions of the experiments, all movement soon stopped at 8000–14,000 p.s.i. (544–953 atm.). The pressure required to stop movement was usually near that which caused visible structural damage (Table 1, p. 508). Movement was restored on release of pressure provided that the treatment had not been too drastic.

3. In the case of one peritrich ciliate (*Epistilis plicatilis*) a moderate pressure (1000–3000 p.s.i., or 68–204 atm.) caused an increase of ciliary frequency, but at still higher pressures the frequency was drastically reduced.

4. There is evidence that behaviour was affected by moderate pressures, quite apart from changes in the intrinsic activity of flagella or cilia. Certain flagellates and ciliates were stimulated to activity by application of moderate pressures, and others became motionless.

5. *Spirostomum ambiguum* was deprived of its ability to reverse or make the avoiding reaction while at high pressure, and at certain pressures the cirri of the hypotrich *Stylonychia mytilus* beat in a wild and uncontrolled way. It is suggested that high pressure affects ciliary co-ordinating mechanisms in various Protozoa.

I am indebted to Dr J. Yule Bogue (Imperial Chemical (Pharmaceuticals) Ltd.) for a supply of high boiling-point sulphur-free liquid paraffin, used in certain experiments described in this and in the following paper. I am grateful to Mr E. Livingstone for most of the cultures, but for certain species to Mr R. Bassindale and Mr A. Chadwick.

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EFFECTS OF HIGH HYDROSTATIC PRESSURES ON *ACTINOPHRYS SOL* (HELIOZOA)

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(*Received 4 July 1957*)

(With Plate 7)

INTRODUCTION

Actinophrys sol Ehrenberg (Heliozoa) is shaped roughly as a sphere, ranging up to about 50 μ in diameter, from which radiate a number of needle-like axopods. Each axopod contains a central filament, or axoneme, which projects inwards to the nuclear membrane (Distaso, 1908; Bělař, 1923). Although it appears likely that the axoneme acts as a supporting rod for the axopod, it seems uncertain how far it extends towards the tip of the axopod in living material. In the closely related *Actinosphaerium* each axoneme consists of a bundle of fibrils of uncertain arrangement (Roskin, 1925; Rumjantzew & Wermel, 1925; Ritter, 1954), and axopods originate as blunt protuberances, in which the fibrils are said to be arranged more diffusely (Roskin, 1925). It is to be hoped that much more information about the fine structure of axopods will emerge from studies with the electron microscope. *Actinophrys* is able to progress slowly along a surface, presumably by extension and contraction of its axopods, but it does not normally appear to bend them. Objects of prey become surrounded in a food vacuole formed either by the spreading of axopods or by the outgrowth of filmy membranes (Sondheim, 1916; Looper, 1928). Two or more *Actinophrys* may fuse temporarily while one of them is feeding or has a food vacuole.

This work concerns the effects of high hydrostatic pressures on *Actinophrys sol*, and in particular on the axopods of this organism.

MATERIAL AND PREPARATION

Actinophrys sol was cultured in Bristol tap water. Cultures were fed weekly with *Colpidium* sp., and were renewed every 6 weeks or less. *Actinophrys* was normally starved for about a week before use, to get rid of food vacuoles. Specimens were mounted in a thin hanging drop in the pressure vessel already described or in a new vessel of the same internal dimensions but with certain extra facilities. The new vessel is made from a larger block of steel, and measures 11·5 × 11·5 × 8 cm. (deep) externally. Channels in the wall allow circulation of water at controlled temperatures; thermocouple leads pass into the central cavity between a pair of concentrically fitting conical washers of polytetra fluoro-ethane, suitably compressed; and a lever, by means of which droplets on the inside of the window can be mixed, passes in through a gland packed with fibre between high-tensile steel washers. The dead space of the vessel was filled with Boots's medicinal paraffin B.P.; in a few experiments an I.C.I. high boiling-point sulphur-free paraffin was used, without any difference in the results.

RESULTS

In each of seventeen experiments one or more specimens of *Actinophrys* were observed (*a*) without applied pressure, (*b*) at a known high pressure, (*c*) after release of pressure. In seven further experiments two or more treatments at high pressure alternated with periods at no applied pressure. In some experiments the length of the axopods was estimated at frequent intervals of time from measurements of the over-all diameter of the organism minus the body diameter; these measurements were made along two axes at right angles to each other. Pressures of 1000 to 11,000 p.s.i. were used. Periods of treatment ranged up to 3 hr., but for 4000 p.s.i. and over they were much shorter and mostly 3–10 min. The temperature was 13·9–20·8° C., with little variation in any one experiment.

Exposure to 1000 p.s.i. (2 hr.) made no visible difference to *Actinophrys*. At 2000 p.s.i. there was a slight and rather doubtful shortening of the axopods. At 3000 p.s.i. the axopods shortened within a minute or two, and a few were withdrawn; most however persisted for the duration of the test (up to 3 hr.). After 1 min. at 4000 p.s.i. some (at least) of the axopods became beaded in appearance and subsequently disappeared. All became beaded and disappeared at 5000 p.s.i. or over (Pl. 7, photos 2 and 3). In favourable cases the beads of each axopod could be seen to be arranged in a straight line along the length of a fine thread; presumably this thread either included or consisted of the axoneme. Soon (within about 1 min. at 6000 p.s.i.) the threads, carrying with them the beads, became bent irregularly; and the beads came to lie closely packed in a zone around the spherical body of the organism, giving the appearance of a halo (photo 4). At this stage the threads were no longer visible. After some minutes at 6000 p.s.i. or over the outline of the body became angular. The contractile vacuole protruded and in some cases continued to grow but failed to discharge.

On release of pressure the halo of droplets immediately drew nearer to the body surface, and appeared to become converted into or replaced by short blunt radial protrusions of the body surface (Pl. 7, photo 5). Within about 2 min. of release of pressure (unless the exposure had been long) these gave rise to the points of axopods (photo 6), which grew out to about three-quarters of their original length within a further few minutes (photos 7–9). If the period at pressure had been so short (up to 1–2 min.) that distinct beaded axopods persisted, these became shortened on release of pressure until only protruding lobes remained, and these in turn gave rise to axopods as before.

The existence of a critical pressure (or range of pressures) at which the axopods became unstable was indicated in two further experiments at room temperature.

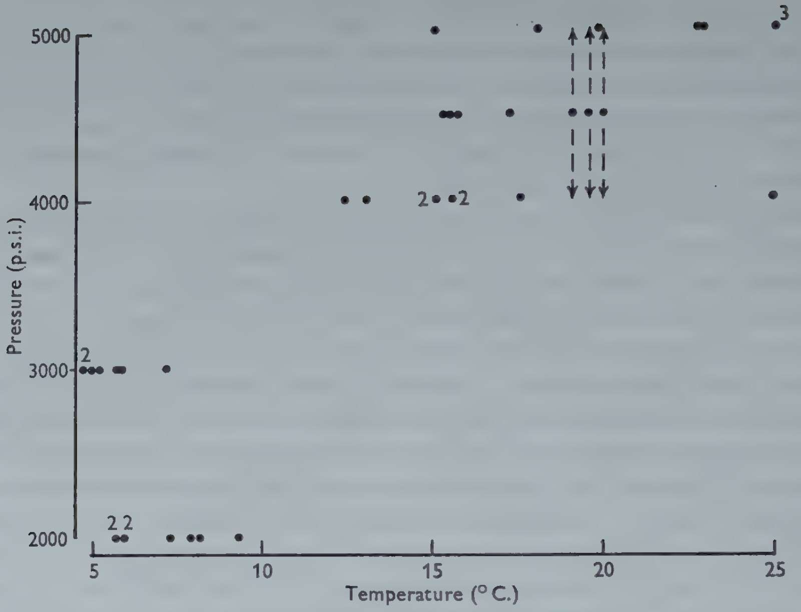
Good axopods were maintained in individuals held at 3000 p.s.i. for 3½ hr., but when the pressure was raised to 4000 p.s.i. the usual changes occurred within 2 min. In individuals in which the axopods had been caused to disappear by a brief treatment at 5000 p.s.i., new axopods began to develop within half a minute of reduction of the pressure to 3000 p.s.i.; however, they failed to reach their full length even during 3 hr. at that pressure.

A further series of experiments was carried out mainly to determine the effect of temperature on the pressure at which the axopods would collapse. In twenty-three experiments the pressure was raised by steps of 1000 p.s.i., with 5 min. at each step. The highest pressure used was varied according to the requirements of the experiment, and after 5 min. at the final pressure, all applied pressure was released to zero in 1–2 sec. The first five of these experiments were carried out with the old vessel at room temperatures of 17–20° C.; the remaining eighteen experiments were made with the new vessel, at temperatures of 4·9–28·5° C., as measured by the thermocouple, including some experiments within the room temperature range.

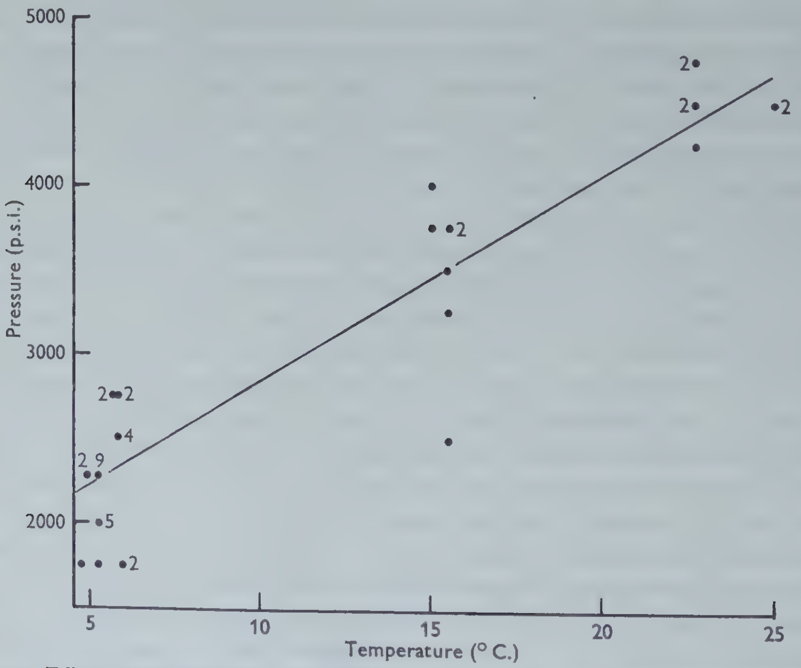
At 17–20° C. there was no obvious change in the appearance of the organisms at pressures up to and including 2000 p.s.i., and little change at 3000 p.s.i. Withdrawal of many of the axopods occurred at 4000 p.s.i., sometimes with some beading, and all had undergone this change before the end of the period at 5000 p.s.i. At this and higher pressures the contractile vacuole stood out from the body surface. Some fusion of food vacuoles appeared to occur in feeding specimens at 9000 p.s.i., and in other cases some food vacuoles were discharged to the outside. (This also happened during prolonged exposure to lower pressures.) Most individuals appeared dead at 11,000 p.s.i., and in fact very few recovered after release from this pressure. All or nearly all recovered on release from 9000 and 10,000 p.s.i. New axopods began to emerge from the surface within 2 min. and were well extended, though not yet fully so, in 5 min. The axopods later extended fully, and the organisms appeared fully normal on the following day.

The effect of temperature on the pressure at which the tentacles all, or almost all, collapsed (in stepwise experiments) is shown in Text-fig. 1. (Results from the first stage of the next series of experiments are also included.) Below 10° C. this collapse occurred at 2000–3000 p.s.i., and above 15° C. at 4000–5000 p.s.i. The differences between individuals were too great to justify any more precise statement; moreover, in any one individual the tentacles did not always all collapse at the same pressure.

An attempt was made at further refinement in twelve additional experiments involving thirty-five specimens. The *Actinophrys* were kept mounted in the new pressure vessel at the experimental temperature for half an hour before an experiment was begun. The pressure was then raised in accordance with standard stepwise procedure until the tentacles collapsed. After completion of 5 min. at this pressure, the pressure was lowered in steps of 250 p.s.i., with 5 min. at each, until the points of axopods appeared. The pressure for reappearance of axopods is plotted against temperature in Text-fig. 2. These results support those previously described. In three of these experiments (at 6–7° and 15° C.), the pressure for reappearance of axopods was examined first by descending steps of 5 min., and then after recompres-



Text-fig. 1. Effect of temperature on the pressure required to cause disappearance of all, or almost all, the axopods of *Actinophrys sol* in standard stepwise experiments. For points marked at 4500 p.s.i. it was uncertain whether the end-point was at 4000 or 5000 p.s.i. Where points coincide, the number of coincident points is given.



Text-fig. 2. Effect of temperature on the critical pressure for restoration of axopods. For details see text. Where points coincide, the number of coincident points is given. The best rectilinear fit is given by $y = 2908 + 121(x - 11{\cdot}0)$ in the units of the graph.

sion by descending steps of 15 min. There was no evidence of any change occurring after the first 5 min. at any pressure, and the results in the two stepwise descents were similar.

In three other experiments, *Actinophrys* was subjected directly to 3000 p.s.i. while at 7–9° C. The axopods disappeared within 2–3 min. The temperature was then raised to 17–19° C. over a period of about 15 min., without change of pressure. Axopods reformed during this period, and became well developed but did not reach their original length while the pressure was maintained.

DISCUSSION

The axopods of *Actinophrys* are capable of elongating and shortening, and this process presumably involves changes in the lengths of the axonemes, although more information is needed about this. It is not known whether any shortening which may occur takes place by extension and contraction or by the local addition or removal of oriented protein. High pressures appear to favour the contraction of protein molecules (Johnson, Eyring & Polissar, 1954, p. 358), so that it is possible to ascribe the shortening of the axopods at 3000 p.s.i. to a contraction of the axoneme or sheath. It would be imprudent to comment further until much more is known about the fine structure of the axopods.

For the interpretation of the effects of high pressures, it is natural to compare the axopods of *Actinophrys* with the pseudopods of *Amoeba*, extensively studied by Marsland. The plasmagel structure of amoebae is broken down at high pressures, and in extreme cases the pseudopods may even break up into separate drops of protoplasm (Landau, Zimmerman & Marsland, 1954). The beading of the axopods of *Actinophrys* upon the sudden application of pressure is probably due to the solation of a gelated layer of the protoplasmic sheath; this outer protoplasm then runs together to form droplets. The loss of rigidity of the axial filament might be due either to the high pressure directly or to an unstabilizing effect of the disorganization or partial loss of the protoplasmic sheath.

When the pressure is released, after an *Amoeba* has become rounded up under the influence of high pressure, a clear layer forms all around the edge and extends progressively inwards. It appears that a structurally organized membrane or gelated layer contracts, carrying with it the granules and other inclusions of the cytoplasm (Kitching, 1954; Landau *et al.* 1954). When *Actinophrys* is decompressed, there is a rapid condensation of the diffuse halo of axopod remains to form the blunt lobes from which new axopods will emerge. It is reasonable to suggest that essentially similar processes are occurring in the two organisms, and that after a period when gelation is inhibited a rapid gelation and gel contraction take place as soon as conditions of pressure permit.

Temperature and pressure act in opposition on the structural and contractile properties of cortical gels, as shown by Marsland and his collaborators. Both *Arbacia* eggs and *Amoeba proteus* stratify (when centrifuged) more quickly at higher pressures and lower temperatures; similarly, cleavage and pseudopod formation are depressed by high pressure, and this effect is enhanced by low temperature. The following are the increments of pressure which are just counteracted by a 10° C. rise of temperature: echinoid egg cleavage, 2000 p.s.i. (Marsland & Landau, 1954); *Rana* and *Chaetopterus* egg cleavage, 1000 p.s.i. (Marsland & Landau, 1954); *Amoeba proteus* pseudopod formation, 2000 p.s.i. (Landau, *et al.* 1954); *Actinophrys* axopod formation, 1200 p.s.i. (this paper).

The outgrowth of a new axopod in *Actinophrys* presumably involves a rather highly organized orientation of protein molecules in the axoneme, and this process takes place quite rapidly; the same is true of the growth of flagella and cilia. The association of the bases of the axial filaments of axopods with the nucleus in certain Heliozoa, and with the centroplast in certain (but not all) others, provides another interesting point of comparison with flagella, although this similarity is probably only of rather broad functional significance. Axopods are incapable of beating, and flagella and cilia are not disorganized by the pressures used in these experiments (Kitching, 1957). It appears that the fibrillar structure of an axopod is much less highly organized than that of a flagellum, that an axopod lacks the specialized excitation apparatus, and that the rheo-plasmic sheath is essentially an elongated pseudopodium.

SUMMARY

1. The axopods of *Actinophrys sol* are caused to collapse by the application of high hydrostatic pressures.

2. Axopods grow out again on release of pressure or on reduction of pressure below a certain critical value.

3. Temperature and pressure act in opposition as regards the outgrowth and maintenance of axopods. At 15° C., the average critical value for the restoration of axopods is about 3500 p.s.i. (207 atm.). An increase of temperature by 10° C. counteracts an increase in pressure of 1200 p.s.i. (82 atm.).

I am indebted to Mr G. A. Shephard (Imperial Chemical Industries Ltd., Alkali Division) and to Mr H. Banwell for much help in the design of the special features of the modified pressure vessel, and to Mr Banwell for making it. The cultures of *Actinophrys* were provided by Mr E. Livingstone.

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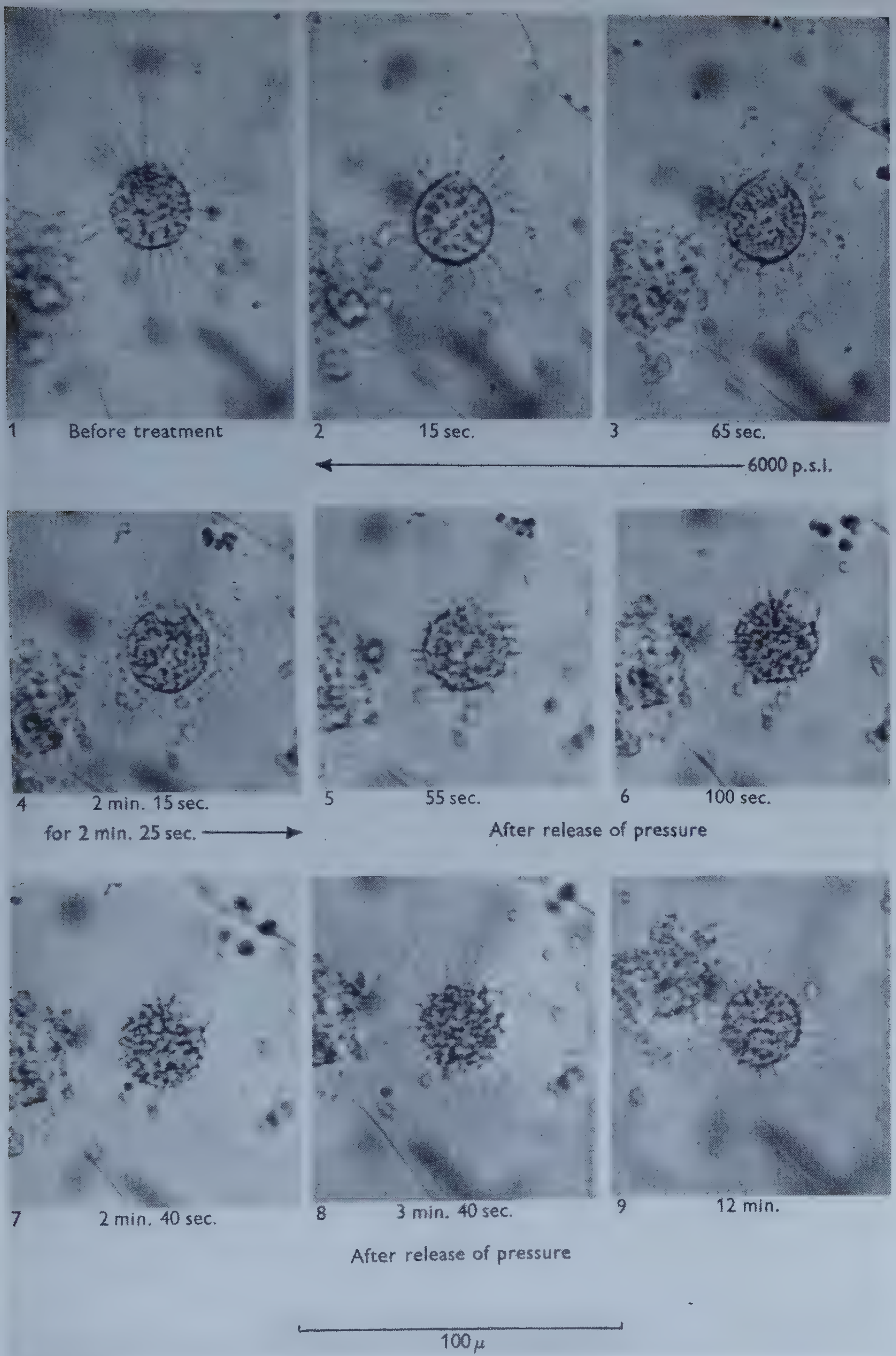

1 Before treatment

2 15 sec.

3 65 sec.

6000 p.s.i.

4 2 min. 15 sec.

for 2 min. 25 sec.

5 55 sec.

6 100 sec.

After release of pressure

7 2 min. 40 sec.

8 3 min. 40 sec.

9 12 min.

After release of pressure

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EXPLANATION OF PLATE

Actinophrys sol before, during, and after exposure to a hydrostatic pressure of 6,000 p.s.i. (408 atmospheres). The axopods became beaded (photos 2 and 3) and collapsed (photo 4). On release of pressure the halo of droplets was replaced by small lobes (photo 5), which gave rise to new axopods (photos 6–9).

TRANSPLANTATION OF MELANOMAS FROM PLATYFISH–SWORDTAIL HYBRIDS INTO EMBRYOS OF SWORDTAILS, PLATYFISH AND THEIR HYBRIDS

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(*Received* 25 *March* 1957)

(With Plates 8 and 9)

The melanomas in the platyfish–swordtail hybrid are produced genetically, as shown by Gordon (1951), by the interaction between the macromelanophore growth-modifying factors from the swordtail and a dominant platyfish sex-linked allele controlling the appearance and distribution of macromelanophores. The genic interaction manifests itself phenotypically in that the macromelanophores appear in the hybrid much earlier than in the normal platyfish and the growth of the pigmented cells seems relatively unchecked, resulting in hyperpigmentation of the animal and ultimately in the production of a tumour. (See Reed & Gordon (1931) and Gordon & Smith (1938) for a detailed description of the phases of tumour development.)

The actions of the macromelanophore genes of the playtfish in the hybrid are manifested in two ways. First, they enable the developing fish to produce macromelanophores and, secondly, they limit the distribution of these cells to relatively localized areas of the fish.

The growth-modifying genes contributed to the hybrid by the swordtail parent may alter the genotype of the pigment cell in such a way that its physiology is basically disturbed, thereby resulting in an overgrowth of these cells; or the effect may be an indirect one on the environment of the growing cells, resulting in hyperpigmentation.

The above suggestions are in certain cases susceptible to experiment. The physiology of the malignant pigment cell has been compared with that of the normal pigment cell in the platyfish in terms of oxidation against selected substrates. Some results obtained by this method have already been reported (Humm & Clark, 1955). In the experiments reported here the environment of the tumour cells has been changed by transplanting melanomatous tissue of the hybrid into hosts of different genetic constitutions and the extent, type, and duration of cell growth in the various hosts have been analysed.

Marcus & Gordon (1954) employed this method and analysed the growth of transplanted *Sc* melanoma cells when implanted under the scales of adult fish of closely related strains of swordtails and hybrids. They compared results from auto-, homo- and hetero-transplantation and reported varying degrees of success in accordance with the genetic relationship between donor and hosts. Except in autotransplants the grafts gradually decreased in size and disappeared. It seemed to us that the failure of homo- and hetero-grafts might be attributed to an immunological reaction on the part of the hosts, rendering it difficult for the tumour cells to maintain themselves in the host tissues. Accordingly, attention was turned to the embryo of the same groups of fish in the hope that grafting at an early stage, before the animals had developed the ability to react immunologically, might allow the tumour to become so firmly established on the host that it would survive after the animal hatched.

The terminology of pigmented cells which will be used here follows that which was adopted during the Third Conference on the Biology of Normal and Atypical Cell Growth (Gordon, 1953).

MATERIALS AND METHODS

The swordtails (*Xiphophorus helleri*) and the platyfish (*X. maculatus*) and their hybrids used in these experiments were bred from an original stock supplied by the Genetics Laboratory of the Aquarium, New York Zoological Society.

Three types of host embryos were used: swordtails, platyfish, and backcrossed swordtail–platyfish hybrids (Text-fig. 1). All were removed by Caesarean section from gravid females.

Swordtail embryos

These were obtained from swordtails which had been inbred for several generations. Their normal pigment pattern results from many widely distributed micromelanophores on their bodies.

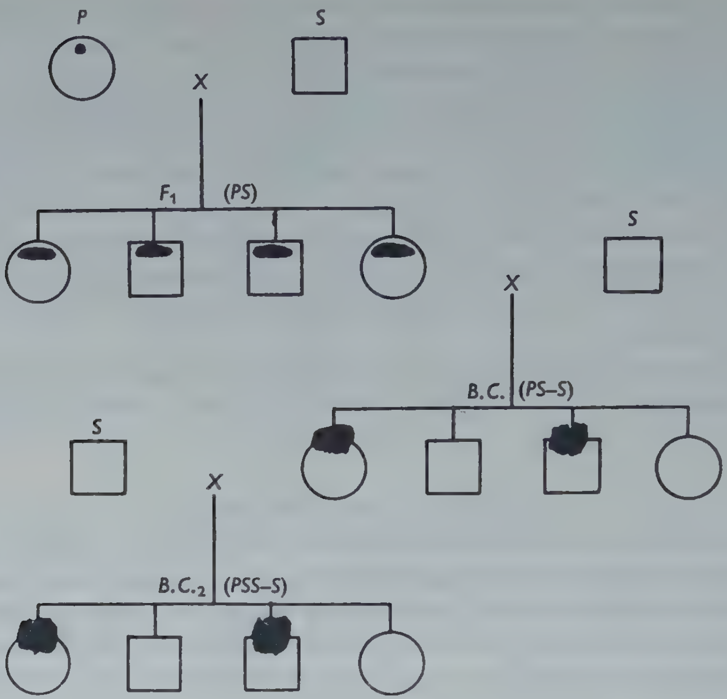
Platyfish embryos

These were obtained from gravid platyfish females of inbred stocks. They, too, were uniformly pigmented by micromelanophores; but in addition had macromelanophores. As Gordon (1948) has shown, one sex-linked allele *Sd* is responsible for the development of macromelanophores in the dorsal fin, another allele *Sp* accounts for the presence of large black pigment cells on the flanks.

Backcross hybrid embryos

The backcross *Sd* embryos (*B.C.*) used were obtained from female swordtails which had been backcrossed with an $F_1(PS)$ male (Text-fig. 1). Only 50% of the backcross hybrid embryos may be expected to develop melanomas because they are the product of a mating between one parent which is heterozygous for the *Sd* allele for macromelanophores and one parent which is recessive for that allele. About 50% of the embryos would be expected to develop into swordtail-like fish and were denoted as *B.C.* (+).

The host embryos were selected at stage 20 using the criteria of Tavolga (1949). The methods of staging, of removing the host embryos from the mother, and decapsulation have been described in a previous paper by Humm & Young (1956).



Text-fig. 1. ○, female; ⊙, spotted-dorsal macromelanophore gene (*Sd*) pattern of platyfish; □, male; ◓, ◓, *Sd*, platyfish gene pattern, the product of interaction with growth-modifying genes of the swordtail; result, melanosis or melanoma in hybrid. *P*, platyfish; *S*, swordtail; F_1 (*PS*), platyfish–swordtail hybrid; *B.C.* (*PS–S*), offspring of an F_1 (*PS*) hybrid and swordtail; $B.C._2$ (*PSS–S*), offspring of a *B.C.* hybrid and a swordtail.

PREPARATION OF THE DONOR TISSUE

Melanoma tissue was obtained almost exclusively from $B.C._2$ *Sd* hybrids. The donor was placed in 1:10,000 merthiolate (Lilly) for 30 min., and a small piece of melanoma removed by means of iridectomy scissors. Under the dissecting microscope the tissue was cut into small pieces and maintained until used in 3 × Niu's solution. Tissue fragments kept for 60 min. in Niu's solution were just as viable as those kept for shorter periods.

Transplantation sites

In the earlier experiments, a small notch was made with glass needles through the ectoderm of the host, just anterior to its dorsal fin, and a $0{\cdot}1$ mm.^{3} piece of tumour tissue was immediately laid into the notch. Attachment was good and the graft healed well into place. There was little tendency for the host tissue to heal over the graft and often part of the tumour fragment was exposed to Niu's solution. This exposure resulted in the disintegration of the exposed portion within 3 or 4 days.

Later, melanoma fragments were placed under the extra-embryonic membranes of the embryos. Turner (1937) and Tavolga (1949) have shown that in the early stages of development the serosa grows up and over the head of the embryo very much like the extra-embryonic membranes of the chick. The serosa consists of a double membrane which is heavily vascularized and thus presents an ideal site for melanoma cells. Just lateral to the eye the inner membrane remains closely applied to the embryo, while the outer membrane bellies out slightly. After making a small cut a piece of tumour tissue was inserted between the two membranes and by means of a gentle stroking with a ball-tipped glass needle the tissue was moved to the dorsal aspect of the embryo's head. Here the melanoma fragment is protected and comes into intimate contact with an abundant circulation. Grafts placed in this position apply themselves to the serosa's upper and lower membranes and spread out in a sheet (Pl. 8, figs. 2–4). These grafts are essentially chorio-allantoic. Continuous microscopic observation and photography of these grafts is facilitated since the fragment is always uppermost in the embryo because its relatively heavy yolk prevents the embryo from turning over. The cellular migrations from the tumour implants are clearly traceable.

The life of such a chorio-allantoic graft was limited. As the extra-embryonic membranes regressed at the approach of hatching the tumour cells growing in the membranes also regressed. Only a few melanoma cells moved away from the membranes and became attached directly to the host.

Two modifications in grafting technique were developed to improve the chances that tumour tissue would attach to the embryo proper and remain after the serosa regressed. (1) A small cut was made through the extra-embryonic membrane just over the opercular region of the embryo. With a pair of Dumont forceps this hole was held open while a small piece of tumour tissue was pressed through the hole and under the operculum of the developing fish. The operation is dangerous since it impinges on one of the larger veins of the extra-embryonic circulation, the rupture of which almost always causes death of the embryo. While the graft did not grow as well or as immediately in the subopercular position some tumour cells did attach to the host and spread. (2) With the embryo held firmly in an agar depression, the tips of the Dumont forceps were pressed under the rear margin of the extra-embryonic membrane, lifting both membranes away from the embryo. A piece of tumour tissue inserted between the slightly spread points of the forceps came to lie against the side of the skull of the embryo where it came into contact with the abundant serosal circulation. Such grafts grew well and as a result large numbers of tumour cells became attached to the surface of the embryo.

The proper maintenance of the post-operative embryos contributed greatly to the success of the experiments. After the insertion of the graft, the embryo was placed in a stendor dish with a minimum volume of growing medium which had the following composition: 3 × Niu's solution to which had been added—10 i.u. penicillin G per c.c., 0·25 mg. dehydrostreptomycin per c.c.; casein hydrolysate (enzymic) to a final concentration of 25 mg. %; 0·02 ml./ml. embryonic extract, 1 : 50 (beef serum ultrafiltrate gives at least as good results).

Formerly, glucose was added to the nutrient medium (Humm & Young, 1956), but its use was discontinued since it appeared to have a slightly deleterious effect on the survival of the embryos.

Despite the presence of penicillin and streptomycin in the culture medium, frequent infections occurred during observations of the embryos which required removal of the stendor dish cover. This hazard was eliminated by sealing a sterile 2 × 3 in. microscope slide to the ground surface of the stendor dish by means of a sterilized mixture of beeswax and vaseline, 1:3. This made an airtight, sterile and optically flat seal through which the embryo could be examined under a dissecting microscope. It was also possible to photograph it with a 32 mm. Micro Tessar lens using paired electronic flash guns as light sources.

After about 30 days, when the yolk mass of the embryo had decreased considerably, the cover-glass was taken off and the hatchling was serially exposed to a decreasing concentration of salts. Several changes of decreasing salt concentration were usually required before the fry could be safely released into tank water. As soon as possible the small fish were fed with brine shrimp nauplii.

RESULTS

All melanoma grafts, regardless of the site of implantation, gave rise to three types of pigmented cells: melanophages, melanocytes and macromelanophores. Of these the first cells to appear on the embryo's surface, singly or in groups, were melanophages which made their appearance around the margin of the graft 1–5 days after the operation. They were small cells, roughly triangular, containing much pigment. These cells migrated very actively, frequently traversing the entire length and surface of the embryo. They finally disintegrated and were eliminated in a manner described by Marcus & Gordon (1954), who studied the fate of these cells after a melanoma fragment was implanted under scales of adult fish. It is presumed that melanin-laden macrophages erupt through peripheral tissues and are lost—a process also described earlier by Gordon & Lansing (1943).

Two types of melanocytes begin to migrate from the margin of the graft during the 1st–5th day after transplantation. Those which first emerge are characterized by having long, slender processes, and by being quite dark and heavily laden with pigment. These melanocytes were similar in appearance and motility to those studied in tissue cultures of fish melanoma by Grand, Gordon & Cameron (1941). The second type of melanocyte, which appears a little later, is light grey in colour, has broader processes and contains local concentrations of melanin around the nucleus and at the periphery of the cell. Both types of melanocytes migrate slowly, spread widely but remain in contact with one another.

The shape of the two types of melanocytes can differ tremendously, depending upon their immediate environment. When crowded the melanocytes assume a long thin condensed appearance, whereas when they are migrating upon the surface of the embryo or in an uncrowded portion of the yolk they will assume the serrated shape typical of broad melanocytes. Plate 8, fig. 1, is a photograph of a whole mount of a melanoma graft and shows thin melanocytes, broad melanocytes and melanophages.

The macromelanophore is a much larger polymorphic cell of 300–470 μ, with lobulated or dendritic processes. It has been described by Grand *et al.* (1941), Ermin & Gordon (1955) as having oval or polymorphic nuclei that are 15–20 μ in diameter; it may contain one or two nucleoli and a loose network of chromatin.

Grafts into the dorsal notch of thirty-five swordtail embryos

The grafts of melanoma tissue into a dorsal notch of thirty-five swordtail embryos suffered two distinct fates. Either the graft broke down immediately into isolated clumps of pigmented tissue and was then attacked by macrophages, or after a portion of the graft had broken down and the cellular debris was removed, macromelanophores appeared, usually under the epidermis; but melanocytes were never seen. After an initial period of spreading, which seldom exceeded a week, the large pigment cells remained static for relatively long periods of time, and then gradually disappeared. Since the cells implanted in this manner were in an ideal position to invade the host tissues and occasionally did, the degenerative changes in the grafted cells cannot be assumed to be due to a poor nutritional environment. Similar cells in the same position on the back of a hybrid fish invade and destroy muscle and connective tissue. It can be concluded that the failure of these cells to grow represents evidence for a basic difference in the tissue environment of the cells. The wild-type swordtail embryo apparently does not present as favourable a growth environment for the pigment cells as do the hybrid tissues.

Grafts into the extra-embryonic membranes

(1) *Swordtail embryos*

The sixty-six grafts placed on the extra-embryonic membranes of the swordtail embryo presented quite a different cytological picture. A high percentage (68%) of the grafts were successful and gave rise to typical melanocytes, usually but not invariably accompanied by macrophages. The growth of these melanocytes continued for a week or more with an occasional case in which it continued for several weeks. After this growth period the graft, although now spread out in a thin sheet on the surface of the membranes, became static. See table 1.

The survival of swordtail embryos bearing tumour grafts depended to a considerable extent upon the size of the graft. The protocols indicated that a distinct antagonism existed between the graft and the host which was manifested in two ways. When the graft was large, it spread out on the host, and within 4–7 days caused a circulatory failure in the host characterized by extensive engorgement and stasis of the blood vessels of the yolk followed by apparent haemolysis and clotting of the blood. This was judged to be an immunological response, and Hildemann (1957) has described a similar circulatory phenomenon resulting after the homotransplantation of scales in goldfish.

When the graft was small, the host destroyed the cells and engulfed the graft; the host embryo continued to grow and eventually hatched. When the graft was neither too large nor too small, the graft initially grew successfully and the host embryo did not suffer undue reaction, but during a period of up to 2 months the tumour cells gradually and completely disappeared. The swordtails continued their development to maturity.

The effect of the grafting procedure on the host was evaluated by sham operations. A hole was made in the embryo's membrane but no piece of tissue was inserted. About 80% survived but were distinctly retarded in their subsequent growth, as compared with the normal development time in the ovary, probably attributable to a considerable extent to the inadequacy of the growth solutions used.

Table 1. *Time of appearance, duration, and disappearance of melanocytes in transplants of fish melanoma fragments from platyfish–swordtail hybrids in the extra-embryonic membranes of swordtail, platyfish and hybrid fish embryos*

Type embryo	I Number	II Melanocytes appearance (average days)	III No. embryos* surviving circulatory crisis	IV Melanocyte growth (average days)	V To average day of degeneration of melanocytes	VI Average survival in days	VII No. ha
Swordtails	66	4·8	32 (48 %)	14·3	22·7	16	8 (25
Platyfish	60	5·7	20 (33 %)	30·5	40·5	44	11 (55
B.C. hybrid	47	8·0	18 (38 %)	24·0	38·0	18	6 (33

* High mortality was experienced in all series between 4 and 7 days due to circulatory stasis and apparent clotting and haemolys blood. Only the animals surviving this crisis are considered in columns IV–VII.

(2) *Platyfish embryos*

Fragments of melanoma were transplanted to the extra-embryonic membrane of sixty platyfish embryos carrying either the *Sd* (spotted-dorsal) or *Sp* (spot-sided) allele or both. Forty platyfish embryos died from circulatory failure between 4 and 7 days following the operation. Eleven of twenty, or approximately one-half, of the host platyfish embryos survived to hatching time, whereas only eight of thirty-two, or one-quarter, of the host swordtail embryos survived under comparable conditions. These results suggest that the platyfish embryo tissues offered a considerably more favourable genetic environment for the growth of melanoma cells than the tissues of the swordtail embryo.

Even more striking evidence of the above was obtained from other details in the protocols. After tumour tissue was grafted onto swordtail embryos, some cells spread out in a thin sheet, but they never seemed to multiply. However, in both the platyfish embryos and, as will be seen subsequently, in the hybrid embryos, the tumour cells definitely proliferated. Several of the host platyfish embryos died from the proliferation of tumour cells which invaded the pericardial area and physically impeded the action of the heart.

(3) *Backcrossed hybrid embryos*

The most striking differences observed between the host hybrid embryos and host platyfish and the swordtails were found in the type of tumour cell migration, proliferation and invasiveness. This may be illustrated by comparing Pl. 8, fig. 4, which shows a graft 9 days after transplantation on to a hybrid embryo, and Figs. 2 and 3 of Pl. 8, which show swordtail and platyfish embryos respectively, bearing malignant grafts of comparable age. The pigment cells in the hybrid show broad processes typical of rapidly migrating melanocytes. In the platyfish and swordtail embryos the pigment cells have barely become established and only thin projections of melanocytes may be seen under the edges of the grafts. The tremendous activity of the melanoma cells killed many hybrid embryos early. In the hybrid shown in fig. 4, the tumour cells multiplied so profusely that they filled the entire pericardial cavity in 7 days and finally killed the host.

A further significant difference between the hybrid embryo hosts and those of others was found in the length of time tumour cells exhibited active migration. Melanoma cells on one hybrid embryo (Pl. 9, fig. 3) after 30 days had proliferated abundantly, migrating and spreading over most of the head and yolk sac. For comparison, in a platyfish embryo of the same age, it may be seen in Pl. 9, fig. 2, that the tumour cells have not proliferated and migrated as extensively as in the hybrid. Compare also the swordtail embryo with a graft of the same age (Pl. 9, fig. 1).

Despite their luxuriant growth in the embryos the melanoma cells did not gain a permanent foothold on the young fry. One graft which had grown very abundantly on the hybrid embryo (Pl. 9, fig. 3), and had come to cover the anterior portion of the body on the left side, continued to grow for 43 days; then it disintegrated quite rapidly as shown in Pl. 9, figs. 4–6. The graft had consisted (fig. 4) of many macromelanophores which were typically chocolate brown and powdery in appearance; this is characteristic of the intact melanoma overgrowth in adult hybrids. Retrogression of the graft coincided with the appearance of six or eight melanophages at the anterior edge of the graft; then the number of melanophages increased rapidly in the next few days. In the meantime individual pigment cells and groups of cells disintegrated, leaving small clumps of pigment granules behind. In about 8 days the whole graft (Pl. 9, fig. 5) consisted of clumps of melanophages and pigment detritus. The disappearance of all graft pigment was complete in 13 days after the first sign of degeneration.

DISCUSSION

Although 101 transplantations of melanoma tissue to swordtail embryos were made, significant growth never occurred in any of them. This observation is in accord with that made by Marcus & Gordon (1954), who reported little or no success after *Sc* melanoma fragments from hybrids were transplanted under the scales of adult swordtails. Thus it would appear that the genetic constitution of swordtails lacks the proper environmental qualities suitable for the growth of transplanted hybrid melanocytes and macromelanophores. The growth of grafted tissue on the platyfish embryo was significantly better than on swordtails but inferior to the melanocyte growth on the backcross hybrid embryos. In ten grafts on hybrid embryo hosts, the rapid and excessive growth of the tissue actually killed the animal by growing into the pericardium and stopping the heart. Only two of the platyfish embryos were killed in this manner, but none of the swordtails. The basic environmental requirements for the survival of malignant melanocytes were present in both the platyfish and hybrid embryos, while the swordtail's environment lacked almost completely the competence to support growth.

The better growth of tumour tissue on the platyfish and hybrid embryos may be accounted for by a consideration of the genetic make-up of the hybrid donor and its tumour as compared with the constitutions of the swordtail, the platyfish, and the hybrid embryos. The hybrid tumour tissues must of necessity possess at least one platyfish chromosome that carries the macromelanophore allele. But owing to the fact that the hybrid embryos were the product of at least one backcross to pure swordtail (Text-fig. 1) the chances are that the backcross hybrid embryos had more swordtail than platyfish chromosomes. But since embryos with a complete complement of 48 swordtail chromosomes do not provide a suitable medium for the growth of platyfish–swordtail hybrid melanomas, it appears that a certain quantity of platyfish heritable material is necessary, probably at least the chromosome carrying the macromelanophore allele. This interpretation is consistent with that of Snell (1953), according to whom the best growth of transplanted tumour tissue should have been obtained (among those hosts employed) in the backcrossed fish. These fish possess a much wider spectrum of inherited cellular antigens than either parent, and the graft by virtue of its own heterogeneity would appear less foreign to this host and be less likely to provoke a transplantation immunity reaction.

In this respect it is interesting to note that the tumour grew much better in the platyfish than in the swordtail. Since the tumour is made up primarily of pigment cells of one type or another, it is interesting to speculate that the possession of the macromelanophore gene 'conditions' the platyfish host somewhat to the reception of the tumour. The logical control, namely the implantation of tumours into platyfish embryos lacking melanophore genes, although projected, has not been performed because of the lack of this strain in our colony.

From other evidence the importance of the platyfish macromelanophore-carrying chromosome has been indicated; thus Marcus & Gordon (1954) had better success in transferring fragments of the *Sc* melanoma into *Sd* dominant *montexumae-helleri* hybrids than to those siblings which were recessive (+). Kallman & Gordon (1957), in experiments in which whole fins were transplanted, concluded that genetic correspondences between donor and host were most important in determining the successful transplantation.

Concerning the effect of hybridization upon the macromelanophore, it appears from other work done in this laboratory (Humm & Clark, 1955; Humm & Humm, unpublished) that an admixture of swordtail genes definitely changes the physiological constitution of the macromelanophore. These changes are manifested by a greatly augmented oxidative capacity in the tumour melanocyte. Whether or not these changes in the metabolism of the cell are causative in the production of hyperpigmentation and eventual melanoma formation will be the subject of further investigation.

Marcus & Gordon (1954), Ermin & Gordon (1955) and Greenberg, Kopac & Gordon (1956) have reported the conversion of a cell having the shape and characteristic pigment pattern of a melanocyte into a pigment cell having the characteristics of a macromelanophore. We have observed similar cellular changes repeatedly. It would appear, however, that the physical character of the substratum is an important determinant of the shape of these cells. When the first migration from a graft occurs it is characterized by the appearance of very long thin dark processes. As these cells migrate out from the graft they begin to spread out and usually fill the space between two small blood vessels on the serosa. The anterior edge of such a cell possesses a fringe of pigment, but the main body of the cell is only lightly pigmented. This type of cell, the broad melanocyte, has been described as a cell type different from the small dark melanocyte; however, we believe that the difference lies in the type of substratum upon which the cell is moving.

When the migration of the graft has continued to the point where the pigment cells have spread out in a single-layered sheet, migration appears to cease. The most spectacular case of this indicates that the cells may remain static for several months. Under these circumstances the pigment cells tend to assume a highly dendritic rounded appearance very typical of the macromelanophore. Accordingly, it seems quite possible that the shape of the three 'types' of pigment cells reported from the melanoma of the fish may be a result of crowding and the substrate upon which they are migrating at the time of observation.

SUMMARY

Small pieces of melanoma tissue were obtained from swordtail–platyfish hybrids and grafted into the embryos of swordtails, platyfish and hybrid embryos.

Several types of cells appeared in all the grafts: melanophages, two kinds of melanocytes, and macromelanophores were observed and described.

It was found that the environment of the grafted tissue played a predominant role in the survival of the graft. Grafts to swordtail embryos survived only a short time, whereas grafts to platyfish embryos bearing the gene for macromelanophores survived significantly longer. It was found that hybridization, with or without the macromelanophore gene, presented a still more favourable environment for the growth and migration of pigmented cells.

In no case was it possible to establish and maintain a permanent strain of tumour cells by embryonic transplantation.

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EXPLANATION OF PLATES

PLATE 8

Fig. 1. Photomicrograph of a portion of a graft which was grown on the serosa of a swordtail embryo, showing melanophages (*m.*), dark melanocytes (*d.m.*) and light melanocytes (*l.m.*). ×430.

Fig. 2. Photomicrograph of melanoma graft in place on the extra-embryonic membranes of a swordtail embryo. Length of time after grafting—12 days. Melanophages (*m.*), melanoma graft (*m.l.*). ×50.

Fig. 3. Photomicrograph of a melanoma graft in place on the extra-embryonic membranes of a platyfish embryo. Length of time after grafting—6 days. ×50.

Fig. 4. Photomicrograph of a melanoma graft in place on the extra-embryonic membranes of a platyfish–swordtail hybrid embryo. Length of time after grafting—9 days. ×50.

PLATE 9

Fig. 1. Photomicrograph of the growth of a melanoma graft on a swordtail embryo 30 days after transplantation. ×75.

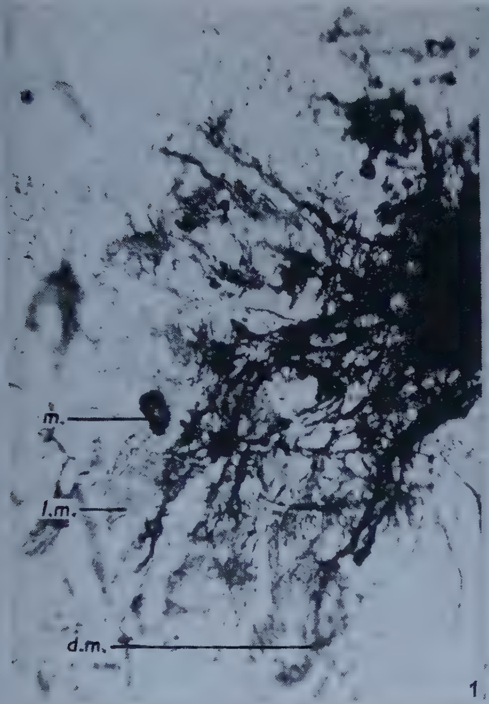
Fig. 2. Photomicrograph of the growth of a melanoma graft on a platyfish embryo 30 days after transplantation. ×75.

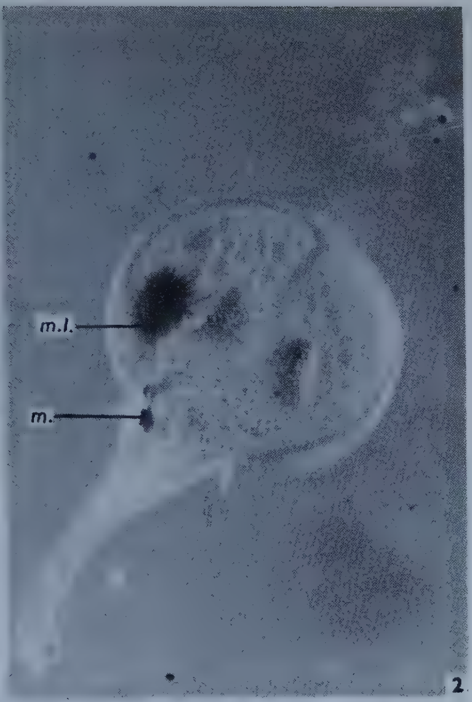
Fig. 3. Photomicrograph of the growth of a melanoma graft on a platyfish–swordtail hybrid embryo 30 days after transplantation. ×75.

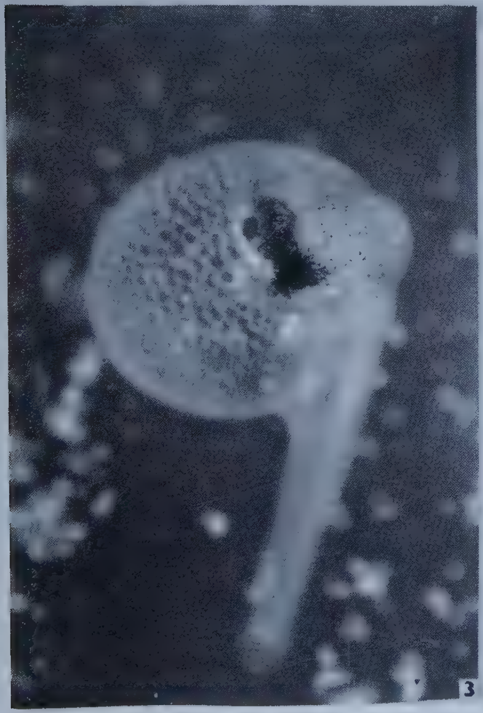
Fig. 4. Photomicrograph of the dorsal aspect of the platyfish–swordtail hybrid embryo seen in fig. 3—70 days after transplantation. ×75.

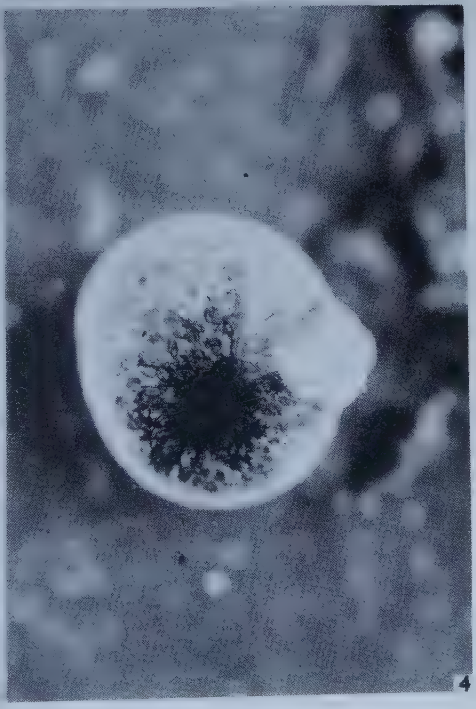
Fig. 5. Photomicrograph of the embryo seen in fig. 4—75 days after transplantation. ×75.

Fig. 6. Photomicrograph of the embryo seen in fig. 4—80 days after transplantation. ×75.



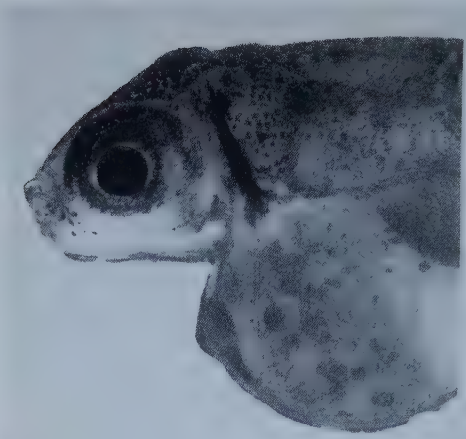






D. G. HUMM, E. E. CLARK AND J. H. HUMM—TRANSPLANTATION OF MELANOMAS FROM PLATYFISH-SWORDTAIL HYBRIDS INTO EMBRYOS OF SWORDTAILS, PLATYFISH AND THEIR HYBRIDS

(*Facing p.* 528)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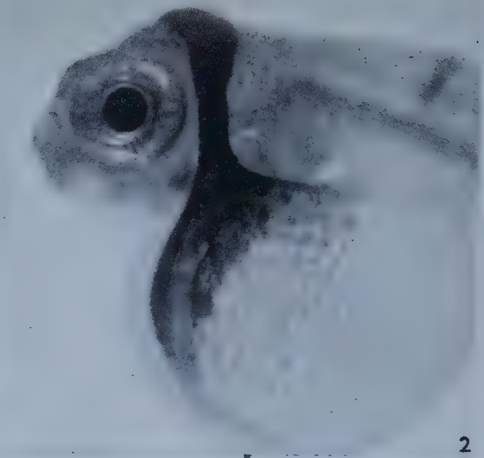
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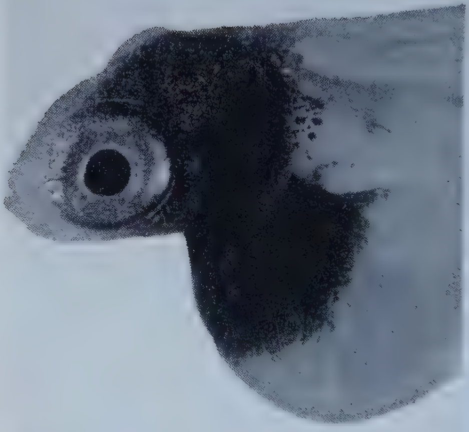
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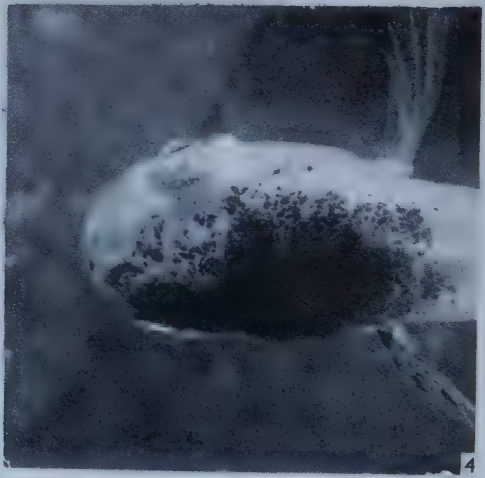
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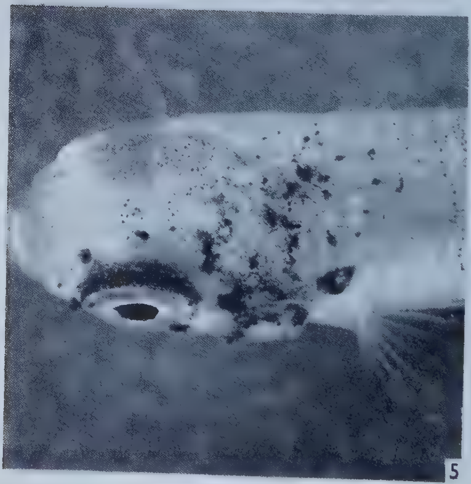
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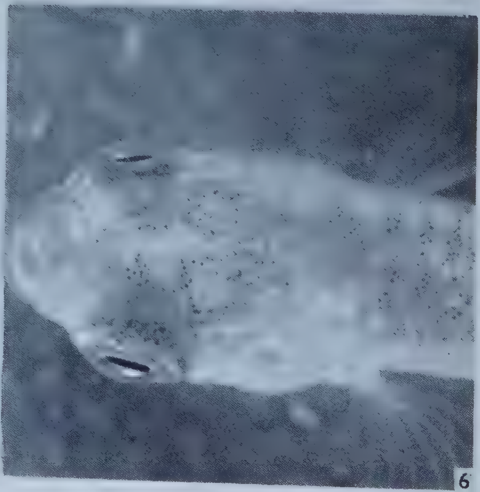
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D. G. HUMM, E. E. CLARK AND J. H. HUMM—TRANSPLANTATION OF MELANOMAS FROM PLATYFISH–SWORDTAIL HYBRIDS INTO EMBRYOS OF SWORDTAILS, PLATYFISH, AND THEIR HYBRIDS

STUDIES ON THE INFLUENCE OF THE EMBRYONIC ENVIRONMENT ON THE POST-HATCHING DEVELOPMENT OF CHICKENS

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INTRODUCTION

In a number of reciprocal crosses between different strains, breeds and species of animals, the two classes of progeny, of both sexes, have been found to differ from one another in the maternal direction. Such maternal effects—clearly not resulting from sex-linked inheritance—must be due to factors either in the cytoplasm of the ovum or in the environment provided for the embryo by the mother. For the purpose of studying the latter the chicken egg offers many advantages. Moreover, such reciprocal differences among chickens, where sex-linkage would in any case be indicated by resemblance to the paternal line, have been reported by Landauer & Dunn (1930), Taylor & Lerner (1939), Hazel & Lamoreux (1947), Moultrie, King & Cottier (1953), Blyth (1953) and Landauer (1953, 1954). Techniques have therefore been developed for replacing a substantial part of the yolk or albumen of chicken eggs with that of another breed or species at an early stage of incubation. These techniques permit a considerable proportion of the embryos to develop and hatch, the post-hatching growth of the experimental birds then being compared with appropriate controls.

Only one account (Bouges, 1930) of experiments involving the exchange of yolk between eggs of different breeds and species of poultry appears in the literature; no conclusive results were obtained. A number of papers have been published reporting work which involved the exchange of albumen. The more extensive experiments include those of Bogolyubsky (1949*a*, *b*, 1950, 1952, 1953), Paspalyev & Bratanov (1950, 1951), Nestorov & Slavtschev (1950, 1953), Nestorov, Slavtschev & Kichev (1955), Hašek (1952*a*, *b*), Mráz & Mrázová (1953), Mráz, Mrázová & Havel (1955), Männel (1954*a*, *b*), Kamiński, Kardymowicz, Piotrowicz & Zebracka-Szczesna (1954) and Letard & Szumowski (1954). These and others have been reviewed in detail elsewhere (Ryle, 1956). Many of the previous workers were attempting to obtain 'vegetative hybrids', i.e. modifications of heredity, but, in the present writer's opinion, none of their results show that this was achieved. Nevertheless, some of their data suggest that exposure to 'foreign' albumen can result in increased body weight, egg production and viability, although, since all the reports are open to various criticisms on technical grounds, these apparent effects of the embryonic environment on post-hatching development cannot be accepted as definitely proved.

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MATERIALS AND METHODS

Donor and recipient eggs were obtained from chickens of several breeds: Legbars (Barred Leghorns), Dorbars (Barred Dorkings), Polish Bantams, Rhode Island Reds and Brown Leghorns of fairly inbred lines, and outbred White Leghorns. Donor eggs from outbred turkeys were also used. In order to have eggs with clean shells, the hens of both donor and recipient breeds of chicken were normally kept in battery cages. They were inseminated artificially once or twice a week. As far as possible a single cock was used to inseminate all the hens of a given breed contributing eggs to a particular experiment. All fowls were fed on the same standard breeders' pellets in order to minimize possible direct nutritional effects of the substituted material. The turkeys had a standard breeders' mixture of a different make. Every egg was marked with the number of the hen, the date of laying and its weight before incubation. At the time of operation the eggs of each hen were arranged according to the date of laying and allocated alternately to the experimental and control groups, with adjustments where necessary to give similar size distributions. (In the yolk exchange experiments the eggs were first candled and those not developing normally were discarded.) Nevertheless, a balanced arrangement of the chicks of each sex seldom resulted. Normally all the eggs of a single hen were operated on before proceeding to those of another. Within each clutch the order of operation of experimental and control eggs was random. Growth comparisons were made only between full siblings of the same sex and, as far as possible, only between those hatched on the same date. Most experiments involved several batches of eggs set on different dates and it proved advisable to accumulate the eggs of each hen for 2 weeks so that a large number could be set simultaneously. The matched groups of chicks were treated as statistical blocks of two treatments: experimental and control. Where more than one experimental (or control) bird occurred within such a group, the mean weight, etc., was adopted as the best estimate of the value for that treatment within the block. The standard errors of the differences between the general experimental and control means (means of group means) were then estimated by analysis of variance. While the rejection, at each stage of comparison, of all birds without any surviving corresponding siblings reduced the numbers drastically, it eliminated between-dam differences, which preliminary experiments suggested were in some cases very large. Where attention is confined only to exactly matched groups, variations due to environmental dissimilarities related to the date of hatching, e.g. maintenance procedure, temperature, etc., are also eliminated.

It was assumed, perhaps unjustifiably, that the earlier the stage at which the embryo encountered 'foreign' material, the more likely was it to be affected by it. It is not normally possible to exchange yolk before 5 days' incubation, as until then most of it is too viscous. At that time Bouges's (1930) technique for eggs incubated 8–9 days is unsuitable, but the following procedure proved satisfactory: the egg was candled and a position marked on the shell in the same latitude as the embryo, in the region of the vitelline blood vessels and about 1 cm. from the edge of the blastoderm. This region was swabbed with 70% alcohol. A small 'window', about 0·5 cm.^{2}, was cut with a fine-toothed hacksaw blade in the marked position. After wiping off the 'sawdust' with cotton-wool moistened with alcohol, the square 'shutter' of shell was gently levered away with the point of a scalpel, and picked up on the free end of a roll of 'Sellotape' $\frac{5}{8}$ in. wide. The underlying shell membrane was made translucent with a drop of medicinal paraffin. After candling again, in order to locate and avoid any blood vessels crossing the window, the egg was placed horizontally with the latter at the highest point. An all-glass 10 ml. hypodermic syringe, held vertically in a sliding holder, was lowered gently until the point of the needle was just above the axis of the egg. If put in deeper it often became blocked with the still viscous portion of the yolk. The appropriate amount of yolk was removed, the syringe withdrawn, and a second syringe containing the same quantity of 'foreign' yolk immediately lowered in the same position into the egg. It was necessary to keep the window at the highest point all the time to avoid air being trapped inside the egg and so preventing reinjection. After injection, the shell 'shutter', stuck on the 'Sellotape', was replaced and the surrounding rim of the latter stuck down on the main part of the shell. The type of egg stand described by Beveridge & Burnet (1946) proved most useful for this work. The eggs were incubated and turned normally both before and after operation. All syringes and needles were washed carefully and sterilized in an oven at 120° C. or more; boiling is inadequate. Scalpels, saw blades, scissors, etc., were flamed frequently during use. The standard quantity of yolk removed and replaced was equal to approximately 5% of the weight of the egg when set, e.g. 2·5 ml. in a 50 g. egg. This proportion is referred to as '5% yolk'. According to Romanoff (1934) and Romanoff & Romanoff (1949), the weight of the yolk increases only slightly by 5 days' incubation and constitutes on the average 32% of the unincubated egg weight. The quantity removed was therefore of the order of 15% of the total yolk volume, though its content of solid matter was undoubtedly less. By 5 days, only part of the yolk has liquefied and 95% of this fraction, compared with about 53% of the remaining dense yolk, consists of water (Romanoff, 1943). After removal and replacement of 5% yolk at 5 days' incubation approximately 50% of the eggs usually hatch. It was found that the unavoidable cooling during operation could be greatly prolonged without increasing embryonic mortality, and it was concluded that this was not an important factor preventing hatching.

Preliminary attempts to exchange albumen in unincubated eggs met with no success: no embryos developed for more than a few days. On learning that other workers had had success after operating at 2 days' incubation, this time was adopted in 1955. In 1956, in order to reproduce more closely the conditions of Bogolyubsky's work, in two of the three experiments the albumen was exchanged after 3 days. The exchange of albumen was carried out as follows: a window about 0·7 cm.^{2} was cut at a position approximately one-third of the length of the egg from the pointed end. The shell 'shutter' was removed on the free end of a roll of 'Sellotape' and the underlying membrane torn away completely with a pair of forceps. The egg was laid in the stand with the axis horizontal and the window directed toward the operator. The syringe, fitted with a very wide bore, stainless steel needle or cannula, about 3 cm. long, was lowered so that the latter passed down between the shell and the yolk. The egg was alternately moved slightly towards the operator and rotated slightly on its axis, as the syringe was lowered further, until the window was at the highest point and the tip of the needle near the lower face of the shell. With this procedure there is little risk of damaging the yolk. It is essential to get the window at the highest point before withdrawing albumen because the air replacing it will otherwise move into the egg away from the opening and form bubbles. These are extremely difficult to get rid of and prevent the replacement of the full quantity of albumen. The albumen was removed and replaced slowly, and the egg sealed in the usual manner and returned to the incubator. Both before and after operation incubation and turning was carried out as for normal eggs. All controls were operated on, having their own albumen put back again. The standard quantity of albumen removed and replaced was approximately equal to 10% of the weight of the unincubated egg, e.g. 5 ml. in a 50 g. egg. This proportion is referred to as '10% albumen'. According to Romanoff (1934) and Romanoff & Romanoff (1949) the albumen has lost little weight by 2–3 days' incubation and constitutes approximately 60% of the total unincubated egg weight. Therefore about one-sixth of the total albumen was removed; this fraction was drawn entirely from the outer thin layer. The donor eggs were mostly infertile, but were usually put in the incubator at the same time as the recipients and were always at the correct temperature before use. Up to 40% of those embryos developing normally at the time of operation subsequently hatched.

After hatching, the birds were at first reared in brooder-house pens with infra-red lamps; later they were moved into folds on grass or into verandah-type pens with slatted floors. Eventually most of the pullets and some of the cockerels were put in battery cages. They were given a standard chick ration for about the first 6 weeks, then standard growers' pellets. Care was taken that the members of each exactly matched group were treated similarly throughout; they were reared together in the same pen until put in battery cages. The chicks were weighed daily at first, then at longer intervals, The shank lengths of most birds were determined before they were discarded, engineer's sliding calipers being used for this purpose. Lack of space as well as high mortalities prevented the collection of much data on egg production by the pullets.

RESULTS

Experiment 1. *Legbars: substitution with Polish Bantam yolk*

On 22 July 1954 twenty-two Legbar eggs were set, of which seventeen proved to be fertile 5 days later. Since no more eggs were available and the mortality among treated embryos was expected to reduce the numbers still further, the control eggs were not operated on. In eleven of the eggs 5% yolk was removed and replaced with that of Polish Bantam eggs which had been incubated for the same period.

Six of these hatched, together with the six controls. One experimental chick had to be killed because of a malformed leg and one control died 3 weeks after hatching. The remaining ten chicks formed, most fortunately, five exactly matched pairs (same parents, same sex) of experimental and control birds. Extracts from their growth data are summarized in Table 1. At first there was no consistent difference in weight between the experimental and control birds, but from 81 days onwards the controls in four of the five pairs were always heavier than the experimental chicks. In the fifth pair there was little difference between the two birds throughout. At 139 days the mean weight of the experimental birds was significantly less than that of the controls ($P=0\cdot05$). At 174 days the difference between the mean shank lengths—0·66 ± 0·243 cm.—just failed to reach the 5% level of significance. One of the cockerels was killed by another bird in the same pen shortly after 174 days, so although the mean weight of the remaining four experimental birds continued to be less than that of their corresponding controls, the difference was not significant.

Table 1. *Growth of Legbars from eggs which received 5% Polish Bantam yolk compared with that of control siblings*

Age (days)	Mean weight (g.)		Difference	S.E. of difference	Difference as % of control mean
	Exp.	Control			
0	39·8	39·4	+0·4	—	1·0
8	70·0	70·8	−0·8	—	1·1
28	299	314	−15	19·3	4·8
49	602	671	−69	46·4	10·3
81	1235	1407	−172	80·6	12·2
139	2056	2404	−348	119·3*	14·5
174	2264	2615	−351	148·4	13·4
200†	2238	2483	−245	109·2	9·9

* Significant at 5% level.
† Only four pairs of birds surviving at this age.

In view of the fact that the control eggs were not treated in any way, the implications of these results are not conclusive. Nevertheless, one might expect any direct effects of the operational procedure to be apparent at, or soon after, hatching—as are those of egg weight—rather than to appear only after 3 or 4 months' growth. The weights of all available Legbar chicks hatched from unoperated control eggs throughout 1954 were compared with those of all control siblings from operated eggs. The former were consistently heavier than the latter at 40 days, but from about 70 days onwards there was some overlap, i.e. just the reverse of the present experiment. Moreover, three exactly matched groups of Legbar chicks were obtained from other yolk exchange experiments, the injected yolk being from Dorbar eggs in one group and from turkey eggs in the other two. Although in the former the control was from an unoperated egg and in the latter the controls were from operated ones, in all three groups the experimental bird (or group mean) was as heavy as, or heavier than, its control at 4 months, and definitely heavier at 7 months, i.e. at approximately 120 and 210 days respectively. This suggested that neither the operative procedure nor 'foreign' yolk, in general, necessarily resulted in retarded growth at the onset of maturity. However, repetition of this experiment with more adequate controls was clearly indicated. Two large-scale attempts were made in 1955 and 1956. Neither was successful, owing to many deaths among the growing birds in both seasons. It should, incidentally, be mentioned that the proportion of the experimental eggs which hatched was greater than that of the operated controls in 1955 and about the same in 1956; thus the bantam yolk did not increase embryonic mortality. Post-hatching mortality was about the same in both groups in each year. In only two of the six matched groups (1/2 in 1955, 1/4 in 1956) surviving to 86 days or more in these experiments did the experimental bird weigh less than the corresponding control: they therefore did not support the 1954 results.

Experiment 2. Polish Bantams: substitution with Dorbar yolk

Seventeen out of forty-one Polish Bantam eggs which received 5% Dorbar yolk hatched, together with 21/43 controls which had their own yolk returned to them.

Table 2. *Growth of Polish Bantams from eggs which received 5% Dorbar yolk compared with that of control siblings*

Age (days)	Mean weight (g.)		Difference	S.E. of difference	Difference as % of control mean
	Exp.	Control			
0	30	30	0	—	0
30	108	97	+11	6·3	11·3
58	317	297	+20	9·2	6·7
72	431	412	+19	8·1	4·6
86	523	506	+17	11·2	3·4
100	629	604	+25	6·3*	4·1
114	721	696	+25	7·9†	3·6
128	781	755	+26	17·8	3·4
142	830	787	+44	40·6	5·6
156	894	824	+70	37·6	8·5

* Significant at 5% level.
† Just fails to reach 5% level of significance.

Twenty-one of these thirty-eight birds were eliminated by death or the lack of corresponding siblings. Among the remaining seventeen there were only three exactly matched pairs; they were therefore divided simply into five groups (i.e. statistical blocks) of full siblings of the same sex. Since the first batch of chicks hatched only a month earlier than the last and the maintenance routine was more constant for the different batches than for those of the larger breeds, the grouping together of siblings hatched on different dates is not without justification. Table 2 summarizes extracts from the growth data. One experimental bird fell sick between 86 and 100 days, and its whole group had therefore to be eliminated, so from 100 days onwards the figures are based on four groups alone. From 58 to 114 days in at least four groups the experimental weight was greater than the control. At 100 and 114 days, as shown in the table, the differences between the general means were significant at the 5% level, or very nearly so. (At 114 days $t=3{\cdot}179$; for $P=0{\cdot}05$ and $n=3$, $t=3{\cdot}182$.) Subsequently, although in three groups the experimental birds maintained their lead, in the fourth it was overtaken by the mean of its four surviving controls. Had it been practicable to keep the birds beyond 156 days it is possible that the difference between the experimental and control means might once again have become significant, for the difference within this group was less at 156 days than at 142 days and, as the table indicates, the value of t increased considerably. The mean shank length of the experimental birds at 156 days was 8·40 cm., that of the controls 8·38 cm. This very small difference indicates that the contrast in body weight was not due to a general increase in all body proportions of the experimental birds.

Several other yolk exchange experiments were attempted, using various recipient and donor breed combinations. In none of them did sufficient matched groups survive long enough to justify statistical analysis; nor were any consistent effects of the substituted yolk apparent. Several chicks, hatched from eggs which received 5% turkey, goose or duck yolk, were reared successfully and appeared in no way abnormal.

Experiment 3. *White Leghorns: substitution with Turkey albumen*

Ten per cent of the albumen of forty-six White Leghorn eggs, in which the embryos developed normally to the 2-day stage, was replaced with that of Mammoth Bronze turkey eggs; twenty-two hatched. Only fifteen of the fifty operated control eggs hatched. Clearly turkey albumen has no adverse effect on the viability of chick embryos. Only five exactly matched pairs of birds were obtained. In contrast with the results of Nestorov & Slavtschev (1950), who found that chicken eggs with turkey albumen hatched later than normal ones, in three of these pairs the experimental chick hatched the day before the control; in the other two it hatched on the same day. In spite of the one day difference, after day 0 the age of all these chicks was regarded as that of the majority of their batch for the purpose of comparing weights, etc. The growth data of the five pairs of birds were examined. In addition, these ten birds and fifteen other healthy ones were divided into nine groups, each consisting of full siblings of the same sex, although not all of the same hatch, and the growth data analysed. By 170 days, in four of the nine groups the (mean) weight of the experimental bird(s) was considerably greater than that of the control(s); in four groups the differences were trivial and in the remaining one the control was definitely the larger bird. Similar variations occurred among the five exactly matched pairs. The general experimental mean for the nine groups at 170 days was $2294 \pm 51{\cdot}2$ g., that for the controls was $2162 \pm 51{\cdot}2$ g. Neither this difference nor that for the five pairs reached the 5% level of significance, nor did any significant weight differences occur earlier in growth. The mean shank lengths at 170 days were almost identical in both cases.

Experiment 4. White Leghorns: substitution with Dorbar albumen

In this experiment four groups of full siblings of the same sex were obtained, including two exactly matched pairs where the birds were of the same hatch. Although in both the latter the experimental bird was heavier than the control, at least from 86 days onwards, the experimental mean of the four groups was not significantly more than the control mean at any age. At 170 days the former was 2420 ± 75·7 g., the latter 2357 ± 75·7 g. The mean shank lengths at 170 days were almost identical.

Experiment 5. White Leghorns: substitution with Brown Leghorn albumen

The percentage hatch was poor in this experiment and no exactly matched pairs were obtained. Comparisons could only be made between siblings of the same sex hatched on widely differing dates and there were only three such groups. While little importance can therefore be attached to the results, it should be noted that, at 170 days, in each group the experimental bird's weight was greater and its shank longer than that of the corresponding control or control mean. In two of the three groups the relatively greater weight became established between 86 and 114 days.

Experiment 6. Brown Leghorns: substitution with Light Sussex albumen

The percentage hatch was again poor and four of the eleven chicks lacked corresponding siblings. The remainder fell into three groups and included two exactly matched pairs. One experimental bird had a badly deformed neck and his growth data are therefore of doubtful value. In one of the remaining two groups the experimental bird was consistently heavier than the control from 19 days onwards, and in the other from 86 days onwards.

Examination of all exactly matched pairs hatched in 1955

Although the experimental mean weight at 170 days was not significantly different from the control mean weight in any one of the last four experiments (all carried out in 1955) it was in each case greater. This was true both where the donor eggs were from other breeds of chickens and where they were from turkeys. In view of this consistent trend, it seemed appropriate to pool the results of all exactly matched pairs of birds, so eliminating environmental differences due to varying hatching dates, and to examine the effects of 'foreign' albumen in general. There were five such pairs in Exp. 3, two in Exp. 4 and two in Exp. 6. One of the latter, however, included the deformed bird and was omitted from the calculations upon which Table 3 is based. In seven of the remaining eight pairs the experimental bird was heavier than its partner at 170 days and the difference between the means, 162 ± 50·5 g. was significant at a level of less than 2%. If the second pair of Brown Leghorns from Exp. 6 is excluded, the difference for the seven pairs of White Leghorns, 170 ± 57·6 g., is significant at almost the 2% level. It is therefore reasonable to conclude from these data that 'foreign' albumen can cause an increase of body weight several months after hatching. The significant difference in hatching

Table 3. *Growth of chickens from eggs which received 10% 'foreign' albumen compared with that of exactly matched controls,* 1955

	Mean weight (g.)		Difference	S.E. of difference	Difference as % of control mean
	Exp.	Control			
Egg wt.	63·1	62·6	0·5	1·04	0·8
Body wt. on day					
0	44·1	39·1	5·0	1·73	12·8*
9	69·5	58·8	10·7	10·0	18·2
19	154	136	18	12·1	12·9
37	377	364	13	21·0	3·6
58	787	759	28	26·9	3·7
72	1091	1050	41	29·5	3·9
86	1377	1338	39	30·9	2·9
100	1633	1557	76	27·5	4·9*
114	1846	1759	87	33·8	5·0*
128	1997	1902	95	42·6	5·0
142	2157	2063	94	61·5	4·6
156	2295	2175	120	63·2	5·5
170	2374	2212	162	50·5	7·3**
Shank length at 170 days, mm.	113·6	112·8	0·8	1·56	0·7

* Difference between means significant at 5% level.
** Difference between means significant at 2% level.

weight, in spite of closely similar mean egg weights, can be accounted for—at least partly—by the three pairs in Exp. 3 where the control chick hatched a day later than its partner. Late-hatching chicks are usually rather lighter than early-hatching ones. From 9 to 86 days, although the experimental mean remained greater than the control, there were considerable fluctuations in individual pairs, as the relatively large standard errors indicate. Between 86 and 100 days the relative difference between the means increased and the variability decreased, so the differences at 100 and 114 days reached the 5% level of significance. The reason for the increased variance from 128 to 156 days is unknown. It will be noted that the difference between the mean shank lengths at 170 days was negligible, although the weight difference was by then 7·3% of the control mean. Thus, as in the second yolk exchange experiment, the greater weight was not due to a general increase in all body proportions, and may have been due merely to more fat or other soft tissues.

Experiment 7. White Leghorns: substitution with Rhode Island Red albumen

Albumen exchange in this (1956) experiment was carried out after 3 days' incubation. The donor eggs were from a very highly inbred strain of Rhode Island Reds. Of the recipients which were developing normally at the time of operation, 24/56 experimental eggs and 25/64 controls hatched; embryonic viability was therefore not reduced by the Rhode Island Red albumen. Thirty-five chicks either died or lacked a corresponding sibling hatched on the same date. The remaining fourteen fell into five exactly matched groups but, even after excluding one group where the experimental bird was, for some unknown reason, abnormally small throughout for a White Leghorn, there was no evidence of a consistent difference in weight between the experimental and control birds. In two groups the former were generally the larger; in the other two the latter were. The mean shank lengths at 157 days were very similar.

Experiment 8. *Legbar × Brown Leghorns: substitution with White Leghorn albumen*

Inbreeding of the recipient breeds was avoided in 1956 in the hope that the viability of the chicks would thereby be improved. As no Brown Leghorn cock, unrelated to the hens, was available, a Legbar cock was used. The eggs were operated on after 3 days' incubation. Twenty-four birds, subdivided into ten exactly matched groups, were obtained. Although from 73 days onwards the experimental mean weight was greater than the control mean, it was never significantly so. At 101, 115 and 157 days in only 6/9, 6/8 and 5/7 respectively of the surviving matched groups was the experimental bird heavier than the corresponding control. At 157 days the difference between the experimental and control means, equal to 6·9% of the latter, was 130 ± 123 g. The mean shank lengths were practically identical.

Experiment 9. *White Leghorns: substitution with Dorbar albumen*

In this experiment—a repetition of Exp. 4—albumen was exchanged after only 2 days' incubation. Embryonic mortality was exceptionally high and eventually only two exactly matched groups of males survived, hatched from eggs of the same hen 3 weeks apart. The single experimental bird in the first group was killed by a stoat shortly after 73 days; up to that age he was consistently heavier than either his own control or that in the second group. The weights of both the other experimental birds, in the second group, were greater than that of either control at 101 and 115 days. However, one of the experimental birds was subsequently overtaken by both controls. The mean shank length of the experimental birds at 157 days was the same as that of the corresponding control.

Thus the results of Exps. 7–9 (1956), separately or jointly, failed to confirm the effect of 'foreign' albumen on growth indicated by the pooled data of the 1955 experiments.

It should be noted that, while some of the previous authors observed certain abnormalities of colour among their experimental birds, in the course of the present work, involving both albumen and yolk exchange experiments, no unusual colouring of plumage, shanks or beak occurred which was not also to be seen among the controls.

DISCUSSION

Regarding Exp. 1 (Legbars; substitution with Polish Bantam yolk), the failure of the limited results obtained in 1955 and 1956 to support those of 1954 remains unexplained. It may merely imply that the latter represented the one case in twenty of such a consistent sample of five pairs of birds occurring by chance, or, alternatively, that the operative procedure itself did in fact have long-term effects. On the other hand, a significant weight difference was also obtained in Exp. 2 where the controls were operated on too. It should also be mentioned that, whereas in 1954 (and 1955) the Legbar chicks were bred from closely related birds, in 1956 care was taken to obtain a cock unrelated to any of the hens. The outbred embryos may have been better able to resist, or compensate for, variations in their environment (cf. Grüneberg, 1954; Searle, 1954; McLaren & Michie, 1954; Biggers & Claringbold, 1954). The Bantams of Exp. 2 were, on the other hand, bred from closely related birds and for this reason may have been responsive to the Dorbar yolk. If the 1954 Legbar result is assumed to be valid, it appears that Polish Bantam yolk can restrict growth of both Legbar and Bantam chicks, under certain (unknown) conditions, since the first grow better in its absence and the second grow better when some of it is replaced with that of Dorbar eggs.

With respect to the albumen substitution experiments, the reasons for the failure of the 1956 results to confirm those of 1955 are also uncertain. It cannot be attributed merely to differences in the degree of inbreeding, for the White Leghorns in Exps. 3–5 (1955) were not from closely related parents. Operation at 3 days' incubation instead of 2 in Exps. 7 and 8 (1956) may have influenced the results; the very limited data of Exp. 9, where the eggs were treated after only 2 days, largely support those of 1955. The trend of the results of Exp. 8 is also in the same direction. It is possible, though improbable, that had there been space to keep the birds longer, more consistent differences would have appeared. In 1955 the difference between the experimental and control means was not significant at 156 days, although it was at 170 days; in 1956 all birds had to be discarded at 157 days. Bogolyubsky and others claim positive effects after operating at 3 days, but it should be pointed out that they exchanged considerably larger fractions of the total albumen than did the present author. The completely negative results of Exp. 7 may perhaps be connected with the highly inbred condition of the hens laying the donor eggs.

If the results summarized in Tables 1–3 are nevertheless accepted as valid, their most interesting feature is the delayed development of any significant effect on weight. This is not apparent until more than 3 months after hatching, about the period of the onset of sexual maturity.* It is, moreover, an effect not extending to the general body dimensions, since shank length differences were usually negligible. Gutteridge & O'Neil (1942) studied the interaction of heredity and environment on growth in poultry. Whereas environmental (climatic and nutritional) differences were clearly reflected in the chick weights from 4–16 weeks, by 24 weeks, i.e. 168 days, only strain differences were significant. These authors state that 'it would seem that as maturity approaches the effect of environment becomes less apparent and the true hereditary size more evident'. In the present work precisely the reverse was true. Significant differences did not appear before 100 days and, in the 1955 albumen exchange work, were more marked at 170 days. Yet the dissimilar environments were confined to the embryonic stage of development.

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* This agrees with Hašek's (1952*a*) observations on ducks which received chicken egg albumen.

Although the results reported here are not conclusive, they suggest that further experimental work on the influence of the embryonic environment on post-hatching growth might yield interesting data. At present one can only speculate on the possible causes of the apparent effects. More analytical work, involving the exchange or injection of particular fractions of the yolk or albumen, is indicated.

SUMMARY

1. Techniques are described for removing and replacing approximately 15% of the total volume of yolk after 5 days' incubation, or of albumen after 2–3 days', in hens' eggs. These allow up to 50 and 40% respectively of the eggs to hatch.

2. Yolk and albumen were exchanged between different breeds of chickens, and chicken eggs received yolk and albumen from other species. Embryonic viability was not reduced by egg constituents of other breeds or species. Small numbers of chickens from eggs which received turkey, goose and duck yolk, and larger numbers from eggs which received turkey albumen, were hatched and reared successfully; they appeared to be quite normal.

3. Legbar eggs received yolk from Polish Bantam eggs. The experimental birds weighed significantly less than their control siblings at 139 days. The mean shank lengths were not significantly different. Attempts to repeat the experiment failed to support this result; possible reasons for this are discussed.

4. Polish Bantam eggs received yolk from Dorbar eggs. At 100 days they were significantly heavier than their control siblings, but their mean shank lengths were almost identical.

5. In each of the four 1955 albumen exchange experiments the mean weight of the experimental birds at 170 days was greater than that of their control siblings, but not significantly so. On pooling the data a significant difference was found at 100 and 114 days ($P=0{\cdot}05$) and 170 days ($P<0{\cdot}02$). The mean shank lengths were very similar. The 1956 experiments failed to confirm these results; possible reasons for this are discussed.

6. It appears that, in certain circumstances, exposure of the embryo to 'foreign' yolk or albumen may influence post-hatching growth in weight, but this effect is only detectable when the chicks are over 3 months old.

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